Behçet's Disease in the United Kingdom:

Genetic Risk Factors and

Ophthalmic Manifestations

SUBMITTED TO QUEEN MARY UNIVERSITY LONDON

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

HARRY JOSHUA DAVID PETRUSHKIN

CLINICAL AND DIAGNOSTIC ORAL SCIENCES

QUEEN MARY UNIVERSITY LONDON

2019

Declaration statement

I, Harry Joshua David Petrushkin, confirm that the work presented within this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Harry Joshua David Petrushkin

Glossary

∞	Infinity
°C	Degrees Celsius
7AAD	7-aminoactinomycin D
ADA	Adalimumab
AIM	Ancestry informative marker
APC	Allophycocyanin
AZA	Azathioprine
B2M	β2 microglobulin
BCE	Before common era
BCVA	Best Corrected Visual Acuity
BD	Behçet's disease
BOSU	British Ophthalmic Surveillance Unit
CAPS	Cryopyrin Associated Periodic Syndrome
CCL3/4	Chemokine ligand 3/4
CCR1	C-C chemokine receptor type 1
CD	Cluster of differentiation
cDNA	Copy deoxyribonucleic acid
CDS	Coding DNA sequence
CE	Common era
CHO	Chinese hamster ovary
CI	Confidence interval
CMO	Cystoid Macula Oedema
CMV	Cytomegalovirus
CNS	Central Nervous System
CTLR	C-type lectin-like receptor
Cy7	Cyanine
DAP12	DNAX-activation protein 12
DMARD	Disease modifying anti-rheumatic drug
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleoside triphosphates
EDTA	Ethylenediaminetetracetic acid
ERAP	Endoplasmic reticulum aminopeptidase
EULAR	European League against rheumatism
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocynate
FMF	Familial Mediterranean Fever
FMO	Fluorescence minus-one
FP	Forward Primer
FRT	Flp recognition target
FUT2	Fucosyltransferase 2
G	Gravity
GA	Georgia
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GOI	Gene of interest
GWAS	Genome wide association study
γδT cell	Gamma delta T cell
HC	Healthy control
HCQ	Hydroxychloroquine
HF	High Fidelity
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
HWE	Hardy-Weinberg Equilibrium
ICBD	International Criteria for Behçet's Disease
IgSF	Immunoglobulin-like superfamily
IL	Interleukin
IMGT	Immuogenetics
IMGT	Immunogenetics
INF	Infliximab
INFg	Interferon gamma
ISG	International Study Group
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
KIR	Killer Immunoglobulin-like Receptor
KLRC4	Killer cell lectin like receptor C4
LAMP-1	Lysosomal-associated membrane protein 1
LB	Luria-Bertani
LD	Linkage Disequilibrium
LogMAR	Logarithm of the Minimal Angle of Resolution
LPS	Lipopolysaccharide
MA	Massachusetts
MAGIC	Mouth and Genital Ulcers with Inflamed Cartilage Syndrome
MC	Mucocutaneous
MCS	Multiple cloning site
MDP	Muramyl Dipeptide
MEFV	Mediterranean Fever gene
MICA	MHC-Class I polypeptide related sequence A
min	Minute
MIP1a/b	Macrophage inflammatory protein 1 α / beta
ml	Millilitres
µl	Microlitre
MMF	Mycofenolate Mofetil
mRNA	Messenger ribonucleic acid
NA	Not available
NEB	New England Biosystems
NF	Nuclease free
Ng	Nanogram
NK	Natural killer
NKG2D	Natural killer group 2, member D

NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NS	Not significant
NSAID	Non-Steroidal Anti-Inflammatory Drugs
ONS	Office of national statistics
OR	Odds ratio
P	Probability
P Blue	Pacific blue
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
Pc	Corrected probability
PCA	Principle Component Analysis
pCMV	Cytomegalovirus promoter
PCR	Polymerase chain reaction
PE	R phycoerythrin
PFAPA	Periodic Fever, Aphthos Stomatitis, Pharyngitis, Cervical Adenitis
PMA	Phorbol 12-myristate 13-acetate
Pred	Prednisolone
QMUL	Queen Mary University of London
RP	Reverse Primer
RPMI	Roswell park memorial institute
rs	Reference single nucleotide polymorphism
rSAP	shrimp alkaline phosphatase
RT	Reverse transcription
SLE	Systemic Lupus Erythematosus
SNP	Single nucleotide polymorphism
SOC	Super optimal broth with catabolite repression
SSOP	Sequence-specific oligonucleotide probe
SSP	Single specific primer
STAT4	Signal transducer and activator of transcription 4
STR	Short Tandem Repeat
STR	Short tandem repeats
TAE	Tris-acetate EDTA
TLR4	Toll-like receptor 4
TM	Trans-membrane
TNFα	Tumour necrosis factor α
TNFAIP3	Tumour necrosis factor α induced protein 3
TX	Texas
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
WHO	World Health Organisation

Abstract

This thesis focuses on clinical manifestations and genetic risk factors in a cohort of individuals with Behçet's Disease (BD) from the United Kingdom (UK).

Chapter 1 outlines the history of BD and discusses the spectrum of clinical presentations. The history of human leukocyte antigen B*51 (*HLA-B*51*) associations in BD is covered and current knowledge of other genetic associations explored. The relevance of the killer immunoglobulin-like receptor 3DL1/S1 (*KIR3DL1/S1*) and its ligand, the Bw4 epitope, is discussed and current knowledge of KIR3DL1/S1 biology is reviewed. **Chapter 2** outlines the materials and methods used in the following chapters. Results are described in **Chapters 3, 4 and 5**. **Chapter 3** explores established genetic associations in BD between *HLA –A* and *–B*, and the MHC Class I Polypeptide-Related Sequence A (*MICA*). Here, the concept of ancestry informative markers (AIMs) is introduced, with a view to reducing heterogeneity caused by population admixture. I confirm the importance of *HLA-B*51* in the UK cohort and in a subgroup made up of individuals of European ancestry. The allele *MICA*009*, is also confirmed as a risk factor for BD, however, its association is likely to be caused by strong linkage disequilibrium to *HLA-B*. **Chapter 4** investigates alleles of *KIR3DL1/S1* and their associations in BD. In this chapter, the construction of 'functional genotypes' to estimate KIR3DL1/S1 expression phenotype is discussed. The combination of a low-expressing KIR3DL1 allotype and KIR3DS1 was found to increase the risk of disease, whereas a high-expressing KIR3DL1 allotype in combination with a KIR3DL1-null allotype decreases the risk of disease. The phenotype of circulating Natural killer (NK) and CD8 T cells is then explored. Low percentages of NK cells were present in PBMC analysed from individuals with BD. Furthermore, KIR3DL1 and KIR3DS1 were expressed at much lower levels than expected. The creation of target cell lines to test the functional effects of the KIR3DL1/S1 'functional genotypes'

is then discussed. I was unable to find any significant differences in degranulation provoked by HLA-B*51, B*52 or B*35^{+} target cells. **Chapter 5** describes the results of a prospective survey of ophthalmic manifestations of BD in the UK. The incidence of ophthalmic BD is 0.04 per 100,000 in the UK. Patient demographics and geography are discussed in detail in this chapter. **Chapter 6** draws together the findings from the Thesis and **Chapter 7** discusses limitations and further work.

Context

In 2008, I was a second-year ophthalmic trainee based in London and keen to travel the world. Prof. Miles Stanford was my clinical supervisor at the time and had mentioned that he had an interest in Behçet's Disease. I wanted to spend some time in the Middle-East and I liked the idea of studying the immunology of Behçet's Disease. Together, we came up with five potential cities in which I could work; Cairo, Damascus, Beirut, Istanbul and Jerusalem. For a variety of logistical, security and academic reasons, Istanbul was the best option. Professor Stanford reached out to Prof Ahmet Gul at the University of Istanbul Medical School in Çapa, who agreed to meet with us. The following year, I travelled to Istanbul to meet the team there. Our research plan was to base my PhD on work previously carried out by Dr. Seema Shafi, one of Prof. Stanford and Dr. Graham Wallace's former PhD students. Seema had focused her attention on various isoforms of MHC class I polypeptide-related sequence A (MICA) in Behçet's Disease, exploring the effects of transfecting various allotypes of MICA into Chinese Hamster Ovary (CHO) epithelial cells. She found that transfecting MICA and human leukocyte antigen (HLA) B*51 and B*52 into CHO cells resulted in provoking differing levels of degranulation from peripheral blood mononuclear cells (PBMC) during co-culture. One hypothesis was that this may have been in part due to differing levels of inhibition provided by KIR3DL1 allotypes expressed on PBMC.

My project would be to investigate allelic variation at the *KIR3DL/S1* locus in Behçet's Disease. As Behçet's Disease is relatively rare in the United Kingdom and *KIR3DL1* is known to be highly polymorphic, we needed to plan to sufficiently power the study in order to have confidence in our findings. Hence the reason for basing the project in Istanbul. Prof Gul has previously published work describing large numbers families with

Behçet's Disease in Istanbul and family-based genetic association studies have significantly more power than case-control studies for rare diseases.

Being an ophthalmologist, I had little laboratory training in medical school. So between 2008 and 2014, I spent a lot of time in the clinical transplantation laboratory in Guy's Hospital under the guidance of Dr. Robert Vaughan and Dr. Emma Lougee, who taught me the basics of molecular biology. I collaborated on a number of research projects, carrying out polymerase chain reactions (PCR), running agarose gels and working on a single-specific oligonucleotide probe (SSOP) Luminex platform. Through this training I was introduced to Dr Paul Norman, who was at the time based in Prof. Peter Parham's laboratory in Stanford, CA, USA and was very helpful in planning my PhD project.

Between 2009 and 2014, life, training, family and parental illness prevented me from starting my PhD abroad and so in February 2014, I registered for a PhD in Queen Mary University London under the supervision of Prof. Farida Fortune, who kindly offered to host my project in her laboratory. This PhD wouldn't have been possible without her support and supervision as well as all the people mentioned above and in the acknowledgments section of this Thesis.

Contents

List of Tables

List of Figures

1 Introduction

1.1 History of Behçet's Disease

Behçet's Disease (BD) is a chronic, relapsing-remitting variable-vessel vasculitis, which can manifest with oral aphthous ulcers, genital ulcers, papulopustular and erythema nodosum-like skin lesions, uveitis, retinal vasculitis, thrombophlebitis, arterial aneurysms, neurological disease, gastrointestinal disease and arthritis. The disease is found primarily along the ancient Silk-Road from the Mediterranean Basin across Asia to Japan (Sakane et al., 1999; Verity et al., 1999).

The constellation of symptoms that make up BD have been described by a number of historical figures from the years 400 before the common era (BCE) -1937 common era (CE) (Figure 1-1). Behçet's disease was introduced to the modern mainstream literature in 1937 by Hulusi Behçet, a Turkish dermatologist and venereologist working in Istanbul Medical Faculty. He described three patients who presented with skin lesions, ocular inflammation and oral ulceration and published his findings in *Dermatologische Wochenschrift* – a German publication, which focused on dermatological disease and manifestations of syphilis. Behçet stated that these patients had no signs of tuberculosis, syphilis or septicaemia and concluded that they were suffering from a previously undescribed condition. He hypothesised that the disease was caused by a dental infection, possibly viral, triggering the observed symptoms. Behçet presented his theory at the Dermatology Association of Paris in the same year. Over the following years a number of cases were reported from Europe, Israel, America and Japan and in 1947, the year before Hulusi Behçet's death, during an International Medical Congress in Geneva the disease was named Morbus Behçet and later Behçet's Disease (Sungnack Lee, 2001).

Despite this, it is unlikely Behçet was the first physician to describe the clinical entity now known as BD.

In the 4th century BCE, Hippocrates of Kos wrote:

…but there were also other fevers, as will be described. Many had their mouths affected with aphthous ulcerations. There were also many defluxions about the genital parts, and ulcerations, boils (phymata) externally and internally, about the groins. Watery ophthalmies of a chronic character, with pains; fungous excretions of the eyelids, externally and internally, called fici, which destroyed the sight of many persons. There were fungous growths in many other instances, on ulcers, especially on those seated on the genital organs. There were many attacks of carbuncle through the summer, and other affections called 'rot'; also large ecthymata, and large herpetic lesions in many instances."- The Third Book of the Epidemion (460-377 BCE), (Hirschberg, 1899).

This constellation of symptoms bears striking similarity to the syndrome now known as BD. Six hundred years later, in 200 CE a Chinese physician Zhong-Jing Zhan described a patient with a less-complete clinical phenotype:

The disease of fox puzzling resembles damage due to cold. [The patient is] silent, desires to sleep but is not able to close their eyes, lying down and standing up restlessly. Sores in the throat are called puzzling, while sores in the yin are called fox-like. There is no desire to eat or drink, and [the patient] is averse to the odor of food. The face and eyes may be red, black or white. If sores develop in the upper region of the body, there will be hoarseness. (Zhang et al., 2013)

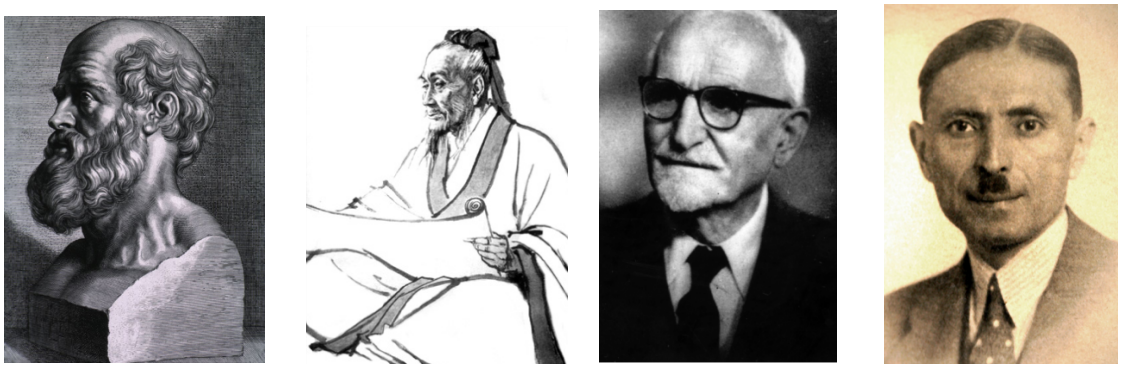
While a number of authors in the 19th century described clinical phenotypes including peripheral arthritis and uveitis, the next clear description of orogenital ulceration in the presence of relapsing hypopyon iritis was described in 1908 by Blüthe, followed by Planner and Remenovsky in 1923 and Shigeta in 1924 (Monacelli et al., 1966).

Seven years prior to Behçet's publication in *Dermatologische Wochenschrift*, an ophthalmologist based in Athens described a patient with uveitis, orogenital ulceration and, the following year thrombophlebitis. Benedictos Adamantiates published his findings in Proceedings of the Medical Society of Athens and Annales d'Oculistique in 1931 and also described how the condition may present as monosymptomatic or oligosymptomatic (Zouboulis, 2002).

Behçet's Disease is a heterogeneous condition and can be challenging to diagnose. In 1990 the international study group (ISG) created the diagnostic criteria for BD (ISG, 1990). To satisfy the ISG definition of BD, patients must present with oral ulceration and at least two other features of BD (Table 1-1). In 2014, the International Team for the Revision of the International Criteria for Behçet's Disease published updated criteria based on a scoring system (ICBD criteria) (Table 1-2).

Figure 1-1. Physicians Credited With Describing The Clinical Entity Now Known As Behçet's Disease

From left to right, Hippocrates (400BCE), Zhang Zhongjing (200CE), Benediktos Adamantiades (1930), Hulusi Behçet (1937)



The criteria was tested across 27 countries and was found to be more sensitive 93.9% vs 81.2% but less specific than the ISG criteria 92.1% vs 95.9% (although this increased to 98.5% in countries which carried out the pathergy test in more than 90% of their patients (International Team for the Revision of the International Criteria for Behçet's, 2014). The rationale for a new scoring system was based around the over-emphasis of oral ulceration in the 1990 ISG criteria. The 2014 ICBD criteria made it easier to offer a diagnosis of BD and to recruit to clinical trials, although the authors emphasised that gold-standard remained an assessment by a clinician experienced in seeing individuals with BD.

Table 1-1. International Study Group Criteria For Behçet's Disease, 1990.

Patients must have oral ulceration PLUS two or more of the minor criteria to fulfil the criteria for diagnosis.

Recurrent Oral Ulceration:	Minor aphthous, major aphthous, or herpetiform ulceration observed by physician or patient, which recurred at least 3 times in one 12-month period.
Minor Criteria:	
Recurrent genital ulceration:	Aphthous ulceration or scarring, observed by a physician or patient.
Eye lesions:	Anterior uveitis, posterior uveitis, or cells in vitreous on slit lamp examination; or retinal vasculitis observed by an ophthalmologist.
Skin lesions:	Erythema nodosum observed by physician or patient, pseudofolliculitis, or papulopustular lesions; or acneiform nodules observed by a physician in post-adolescent patients not on corticosteroids.
Positive Pathergy test	Read by a physician at 24 - 48 hours

Table 1-2. The International Criteria For Behçet's Disease, 2014.

A score of four or more is required for a diagnosis of Behçet's Disease.

Sign/Symptom	**Score**
Ocular lesions	2
Genital aphthosis	2
Oral aphthosis	2
Skin lesions	1
Neurological manifestations	1
Vascular manifestations	1
Positive pathergy test	1

1.2 Behçet's Disease: Clinical Manifestations

As mentioned above, BD offers diagnostic challenges and is difficult to manage. There are no laboratory tests, biomarkers or imaging studies that offer a definitive diagnosis. There is significant geographic variability in the clinical phenotype of the disease and as such, generalisations regarding age, ethnicity and sex must be made with caution.

Behçet's Disease is a variable-vessel vasculitis, which affects most organ systems. Patients classically have a history of oral aphthous ulceration (often from childhood) and develop associated symptoms between the ages of 20-30 years. Several times a year a patient may experience a 'flare' at which point their mucocutaneous manifestations may worsen and other systems may become involved.

Typically, BD is a condition affecting younger patients and it is relatively unusual for patients over the age of 60 years to present with severe symptoms. It is currently unclear why some patients are only affected by mucocutaneous manifestations of the disease, whereas others develop neurological, ocular or gastrointestinal disease requiring long periods of immunosuppression.

1.2.1 Orogenital Ulceration

Orogenital aphthae are a hallmark feature of BD. Oral ulceration occurs in 97-100% of patients (Yurdakul et al., 2008) and can occur in three forms (Taylor et al., 2014):

1. Minor aphthae – less than 10mm, can occur in isolation or in clusters. Healing takes between 7-10 days. Periods of ulceration are typically followed by ulcer-

free periods of varying length. Minor aphthae can affect the labial and buccal mucosa as well as the tongue and oropharynx.

2. Major aphthae are larger than 10mm in diameter and affect deeper tissues. Healing can take up to a month and often leaves significant scarring.
3. Herpetiform ulcers are tiny clusters of ulcers less than 1mm in diameter and are often associated with severe pain.

Pharyngeal ulceration may be mistaken as tonsillitis and patients can be misdiagnosed for some time before other features of BD clarify the diagnosis (Lehner, 1977).

Differentiation between recurrent aphthae in BD from the ulceration seen in other associated diseases is often difficult for the following reasons:

1. Aphthae may precede a definitive diagnosis of BD in up to 71% of patients (Dilsen et al., 1993).
2. Aphthae may be absent at presentation of systemic disease (Shimizu et al., 1998).
3. The systemic manifestations of BD are not idiosyncratic and occur in a number of other diseases involving recurrent oral ulceration.

Genital ulceration occurs in 57–93% of patients (Zouboulis, 1999). The ulcers are similar in morphology to oral aphthae but occur less frequently and often scar. These ulcers cause significant morbidity due to pain in urinating and walking. The scrotum is the most commonly affected area in men, although ulceration can occur anywhere on the shaft or glans of the penis as well as in the perianal area. In women, ulceration most commonly affects the vulva and labia, but can be found in the vagina and cervix. There are a number of other causes of orogenital ulceration (Table 1-3) including drug reactions, viral

infections and neoplasm, so a detailed history must be taken before a diagnosis of BD can be made on the basis of orogenital ulceration.

1.2.2 Dermatological Disease

Patients with BD may present with pseudofolliculitis, pustulosis and erythema nodosum. These are non-specific signs and can occur in a number of other disease processes. In addition, adult-onset acneiform lesions can occur, typically on the upper back and are more common in men.

A positive pathergy reaction is part of the ICBD 2014 diagnostic criteria (although only if performed in >90% of patients). The pathergy reaction is caused by a neutrophilic inflammatory response induced by a sterile needle prick and is read at 24-48hrs (Sobel et al., 1973). If a raised erythematous papule >2mm in diameter is present, then the test is positive.

Historically, a positive pathergy reaction was considered pathognomonic of BD (Katzenellenbogen et al., 1965), however, there are a number of other conditions that can also cause positive results such as pyoderma gangrenosum, chronic myeloid leukaemia, rheumatoid arthritis and Crohn's disease (Varol et al., 2010).

Table 1-3. Causes Of Orogenital Ulceration

There are a variety of causes of orogenital ulceration. The following table is adapted from (Keogan, 2009).

Causes of oral ulceration and orogenital ulceration.	**Recurrent oral ulcers – genital ulceration not reported or very rare**	**Recurrent oral or orogenital ulceration**
Idiopathic	Recurrent oral stomatitis Periodic Fever, Aphthous Stomatitis, Pharyngitis, Cervical Adenitis (PFAPA) Familial Hibernian fever	Complex aphthosis
Multi-system, immune-mediated disease	Systemic Lupus Erythematosis (SLE)	Behçet's syndrome Reiter's syndrome Mouth and genital ulcers with inflamed cartilage (MAGIC)
Gastrointestinal	Ulcerative colitis Coeliac disease Other malabsorption states	Crohn's disease
Nutritional	Iron B12 and folate deficiency	
Dermatological		Sweet's syndrome Erythema multiforme Bullous skin disease Erosive lichen planus Fixed drug reaction
Infection	Human Immunodeficiency Virus (HIV) Chickenpox Hand, foot and mouth disease	Cytomegalovirus (CMV) (in immunocompromised patients) Herpes simplex virus (HSV1)
Drug reaction	Nicorandil (anal ulcers also reported) Bisphosphonates	Non-steroidal anti-inflammatory drugs (NSAIDs)
Haematological		Cyclical neutropenia Lymphoma

1.2.3 Rheumatological Manifestations

Joint involvement in patients with BD has been reported to occur in 11-93% of patients (Caporn et al., 1983; Salvarani et al., 2007). The wide range in frequency is likely to be due to differing diagnostic criteria and natural patient variation across the world. In a recent study from Iran involving 2,312 patients, the authors reported rheumatological complications in 50% of individuals over 5 years (Fatemi et al., 2015).

Typically, patients present with a non-erosive inflammatory monoarthritis. A number of joints may be involved; most commonly the knee (34%), ankle (26%) , elbow (11%) and wrist (10%) (Fatemi et al., 2015). Behçet's disease-related arthritis can be self-limiting and last from weeks to months.

1.2.4 Neurological Disease

Neuro-Behçet’s disease can either manifest as parenchymal or vascular disease (Table 1-4). Parenchymal involvement can often be mistaken for other diseases (such as demyelination) and can occur anywhere from the basal ganglia to the pyramids and spinal cord. Patients with vascular disease may present with symptoms of raised intracranial pressure due to cerebral sinus thrombosis, or develop aneurysmal disease and arterial occlusion. Patients may also present with meningitis or meningoencephalitis.

Table 1-4. Neurological Manifestations Of Behçet's Disease

Neuro-BD is typically classified as either parenchymal or non-parenchymal. Amended from (Al-Araji and Kidd, 2009)

Central nervous system
Parenchymal • Brainstem • Diffuse ("brainstem plus") • Spinal cord • Cerebral • Asymptomatic ("silent") Non-parenchymal • Cerebral venous thrombosis: intracranial hypertension • Intracranial aneurysm • Extracranial aneurysm/dissection
Peripheral nervous system (relation to Behçet's disease uncertain)
• Peripheral neuropathy and mononeuritis multiplex • Myopathy and myositis
Other uncommon but recognised syndromes
• Acute meningeal syndrome • Tumour-like neuro-Behçet's Disease • Psychiatric symptoms • Optic neuropathy

Central nervous system (CNS) involvement carries a poor prognosis, usually appears late in the disease and follows a relapsing and remitting course in up to 40% of cases. It is usually considered a rare manifestation of BD occurring in 1% of cases (Tursen et al., 2003).

1.2.5 Vascular Disease

Both arterial and venous disease can occur in BD and vascular involvement is the leading cause of mortality in BD (Saadoun et al., 2010). Large vessel involvement has been

reported in 1.8-39.4% of patients (Bang et al., 2001; Duzgun et al., 2006). Vascular involvement of BD is characterised by aneurysm and pseudoaneurysm formation in arteries and thrombotic occlusion in arteries and veins (S. S. Yang et al., 2013). In a survey of 1,200 patients in Turkey, 14% of patients had venous involvement (mostly venous thrombosis) and 1.6% had arterial involvement, including aneurysms of the femoral, abdominal, popliteal and pulmonary arteries (Kuzu et al., 1994). A more recent study in China retrospectively analysed 796 patients with BD. They found 13% of patients had vascular involvement and in 28% it was their initial presenting feature. The authors also found that 20% of patients with vascular involvement had cardiac disease, compared to 3% of BD patients with no documented vascular disease (Fei et al., 2013).

1.2.6 Ocular Disease

The frequency of ocular BD varies around the world. In some ethnic groups, inflammatory eye disease occurs in over 50% of patients (Yazici et al., 2007). Uveitis is the most common ocular manifestation and can be present in 90% of patients (P. Yang et al., 2008). Behçet's uveitis can be challenging to treat and up to 90% of untreated patients will lose all vision in both eyes within 4 years (Benezra et al., 1986). One of the current epidemiological challenges in BD is to understand better the frequency of ophthalmic manifestations across the globe. There is currently no data describing the incidence and phenotypic range of ophthalmic disease within the United Kingdom.

Behçet's disease can affect the anterior structures of the eye and result in anterior uveitis with relapsing hypopyon and raised intraocular pressure. If there is no posterior segment involvement, these patients can be managed with topical treatment and close observation.

Posterior segment involvement can result in vitritis, retinal vasculitis, retinal infiltrates, optic disc swelling and retinal vascular occlusion. Posterior uveitis in BD can be sight threatening and should be managed urgently. Unless the inflammation is brought under control in a timely manner, anterior and posterior synechiae, cataract and rubeosis iridis with secondary glaucoma will occur. In patients with recurrent vascular occlusions secondary pipe-stem fibrosis occurs, leading to an ischaemic retina and a pale optic-disc. End-stage disease leaves patients with no perception of light in either eye.

While there have been significant advances in the field of immunotherapeutics over the past 30 years, a significant number of patients continue to lose vision because of Behçet's uveitis. In 2008, a study of 400 patients with BD in China reported that over 20% of eyes still became legally blind despite 'aggressive treatment' (P. Yang et al., 2008).

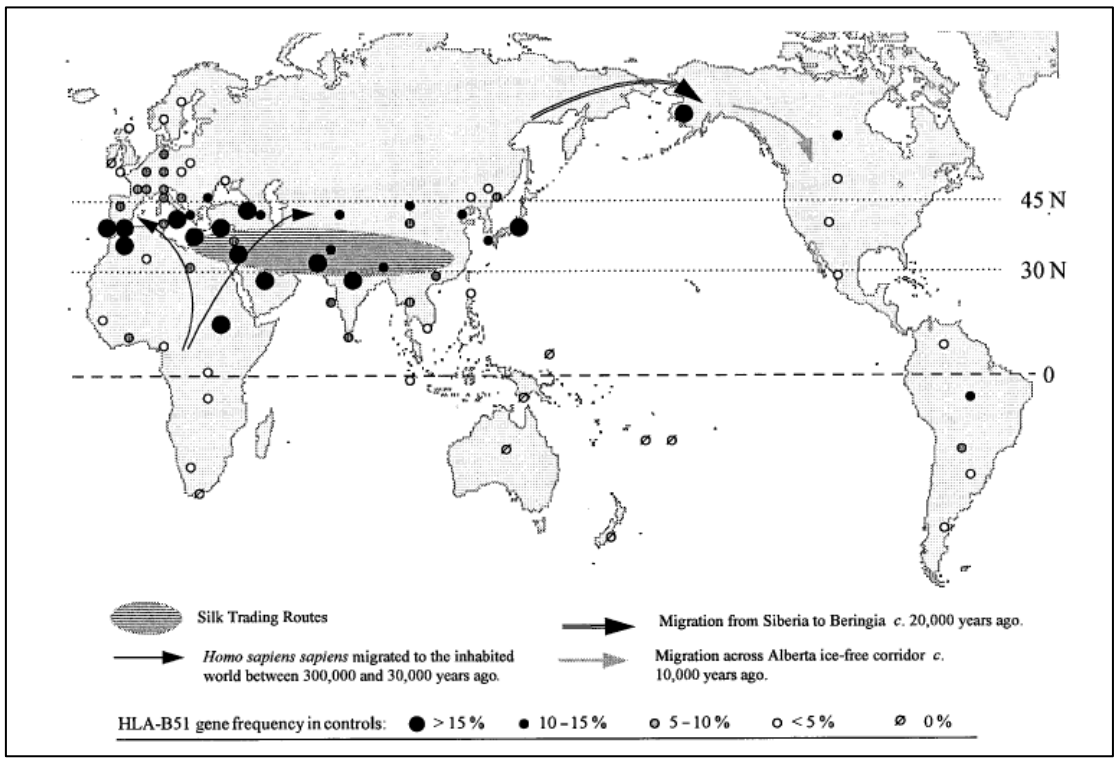
1.3 Behçet's Disease – Epidemiology

Behçet's Disease was labeled the 'Silk-Road Disease' by Shigeaki Ohno in 1982 (Ohno et al., 1982). This was based on the observed disease prevalence in the region between Japan and the Middle East between 30-45 degrees latitude (Figure 1-2).

The prevalence of BD varies across the world (Figure 1-1) and is significantly lower in countries that lie outside the path of the ancient Silk-Road. In the UK, prevalence has been estimated to be 0.64 per 100,000 (Lehner et al., 1979), although recent data from our unit suggest that a more accurate estimate is nearer 2 per 100,000 (unpublished data). By comparison, a prospective study in 2003 estimated the prevalence of BD in Istanbul to be as high as 420 per 100,000 (Azizlerli et al., 2003).

Figure 1-2. Map Displaying Human Leukocyte Antigen (HLA)-B*51 Allele Frequency Across The World And The Location Of The Silk-Road

*Possession of HLA-B*51 is concentrated across the ancient Silk-Route. Migration routes across the Bering Straits led to the appearance of HLA-B*51 in North and eventually, South America. Despite similar levels of HLA-B*51 frequency in some areas of South America to those in Central Asia, the prevalence of Behçet's Disease is significantly lower. Reproduced with permission (Verity et al., 1999)*



As can be seen in Table 1-5 and Figure 1-2, *HLA-B*51* carries a strong geographic correlation with BD. Whether *HLA-B*51* is directly involved in the pathogenesis of BD, or whether it is correlated with the disease due to strong LD with another causative allele remains to be determined (E. H. Hughes et al., 2005; T. Hughes et al., 2013; Kirino et al., 2013; Ombrello, Kirino, et al., 2014; Remmers et al., 2010).

Behçet's disease is thought to occur due to an environmental trigger in genetically predisposed individuals. It is a complex condition and not fully explained by a single gene mutation. A small group of individuals have been identified with a Behçet's-like disease

(Zhou et al., 2016), which behaves in a monogenic Mendelian-manner and arises from pathogenic variants of the tumour necrosis factor α induced protein 3 (TNFAIP3). When assessing individuals with BD-like symptoms it is important to consider other autoinflammatory diseases that may present with a BD-crossover clinical phenotype (e.g. Cryopyrin associated period syndrome (CAPS), or Familial Mediterranean Fever (FMF)). These individuals respond well to anti-interleukin 1 monoclonal antibody therapy and are managed quite differently to those with BD (Lopalco et al., 2015).

Differences in disease prevalence among recent migrants compared with those residing in their home country help establish a role of the environment, while differences in disease prevalence among individuals of different ancestries residing in the same region reflect the role of genetics in disease pathogenesis (Takeuchi et al., 2015). For instance, the prevalence of BD is reduced among Turks who recently immigrated to Germany (15.1 per 100,000) compared with those residing in Turkey (80-420 per 100,000), but is nevertheless high compared with individuals of German ancestry who live in Germany (0.30 per 100,000) (Papoutsis et al., 2006).

1.4 The Genetic Basis For Behçet's Disease

There is some evidence of familial aggregation in BD, which supports the involvement of genetic factors in its pathogenesis (Gul et al., 2000). A study examining a Turkish population reported that 4.2% of siblings of individuals with BD fulfil the ISG criteria for BD themselves (Gul et al., 2000). Aggregation varies with ethnicity and is highest amongst Turks (18.2%) followed by Koreans (15.4%) and Jews (13.2%). Patients of Chinese and Japanese extraction have lower rates of familial aggregation (2.6 and 2.2% respectively) (Fietta, 2005; Zouboulis, 1999). Stronger familial aggregation was observed

among early onset BD patients compared with individuals with disease onset in adulthood (Kone-Paut et al., 1999). Whether this tendency toward familial aggregation in younger patients represents a spectrum between the dominantly inherited TNFAIP3-related disease (Zhou et al., 2016) and sporadic BD remains to be seen.

Table 1-5. Prevalence Of Behçet's Disease Across The World And The Allele Frequency Of *HLA-B*51* In Patients And Controls.

*Behçet's Disease typically presents between 30-45° latitude. This table shows populations at various latitudes and their HLA-B*51 allele frequencies. Adapted from (Piga et al., 2011). BMTR – UK Bone Marrow Transplant Registry. AF – Data available on www.allelefrequencies.net*

Latitude (°N)	**Region (country)**	**Prevalence per 100,000**	**Mean Freq. *HLA-B*51* in controls (%)**	**References**	**Freq. *HLA-B*51* in BD (%)**	**References**
56	Scotland (UK)	0.27	3.9	(Bodmer, 1996)	13	(Jankowski et al., 1992)
53	Yorkshire (UK)	0.63	7	BMTR	18	(Chamberlain, 1977)
52	German (Berlin, Germany)	0.55	12	(Kotter et al., 2001)	36	(Zouboulis et al., 1997)
52	Turkish (Berlin, Germany)	20.75	25	(Kotter et al., 2001)	75	(Zouboulis et al., 1997)
44	Reggio Emilia (Italy)	3.8	18	(Capittini et al., 2009)	57	(Ambresin et al., 2002)
42	Chinese	14.0	24	AF	56	(Mineshita et al., 1992)
41	Istanbul (Turkey)	420.0	15	(Arnaiz-Villena et al., 2001)	NA	(Azizlerli et al., 2003)
41	North-East Turkey	370.0	34	AF	26	(Yurdakul et al., 1988)
41	North West Turkey	20.0	28	AF	28	(Cakir et al., 2004)
40	Spain	7.5	10	AF	37	(Riera-Mestre et al., 2010)
43	Hokkaido (Japan)	30.5	24	AF	28	(Mizuki et al., 1999)
37	Japan	13.5	14	(Ikeda et al., 2015)	59	(Kaburaki et al., 2010)

37	Korea	7.3	9	(Yoon et al., 2010)	53	(Park et al., 2002)
33	Iraq	17.0	27	AF	62	(Al-Rawi et al., 1986)
32	Israel	15.2	8	AF	29	(I. Krause et al., 2007)
32	Druze (Israel)	146.4	12	AF	100	(I. Krause et al., 2007)
32	Arab (Israel)	26.2	43	(I. Krause et al., 2007)	81	(I. Krause et al., 2007)
32	Jewish (Israel)	8.6	38	(I. Krause et al., 2007)	72	(I. Krause et al., 2007)
32	Alexandria (Egypt)	7.6	7	(El Menyawi et al., 2009)	58	(El Menyawi et al., 2009)
26	Al Quassim, Saudi Arabia	20.0	26	AF	72	(al-Dalaan et al., 1994)

1.4.1 HLA Nomenclature

HLA nomenclature changed significantly in 2010. Currently, each HLA allele name has a unique number corresponding to up to four sets of digits separated by colons (Figure 1-3). The length of the allele designation is dependent on the sequence of the allele and that of its nearest relative. All alleles receive at least a four-digit name, which corresponds to the first two sets of digits. Longer names are only assigned when necessary. There are a number of suffixes which can also be added to an HLA type (Table 1-6), these suffixes indicate the expression of the particular HLA in question (Robinson et al., 2015; Robinson et al., 2001).

1.4.2 HLA Associations In Behçet's Disease

Over the past 30 years, HLA nomenclature has become more sophisticated to allow for the huge allelic variation present across HLA. When Ohno et al. first discovered the association between HLA and BD, his paper reported "an association with HL-A5" (Ohno et al., 1973). Over the following decades this association has become more specific. At present, the strongest HLA association in BD is *HLA-B*51:01:01* although in many papers it is referred to in lower resolution as *HLA-B*51*. The *HLA-B*51* association has become recognised as the most frequently observed genetic factor in BD and is present in between 13-80% of patients depending on ethnicity (Gul, 2011).

Figure 1-3. Current HLA Nomenclature System

The current nomenclature was modified in 2010 and is explained below.

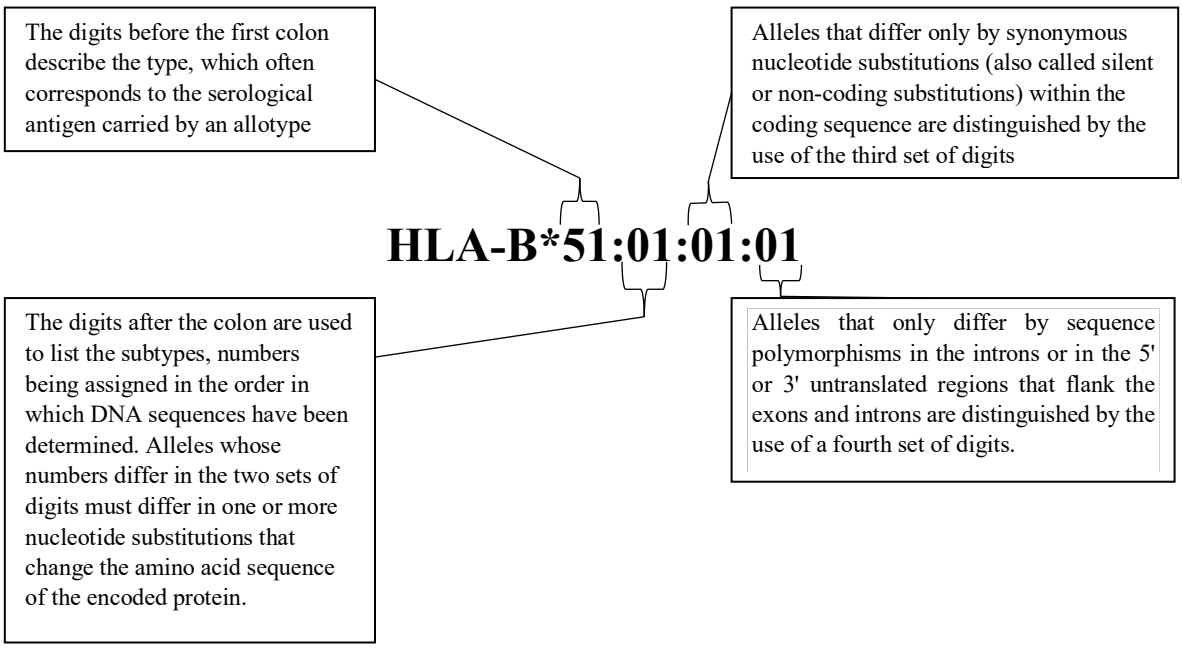


Table 1-6. Suffixes In HLA Nomenclature

There are a variety of suffixes that can be used to describe the expression of HLA allotypes

Suffix	Meaning	Explanation
N	Null	No surface expression
L	Low	Low surface expression compared to normal levels
S	Secreted	Secreted, but not present on cell surface
C	Cytoplasm	Present in the cytoplasm but not the cell surface
A	Aberrant	There is some doubt as to whether the protein is expressed
Q	Questionable	Questionable expression given that the mutation seen in the allele was previously thought to affect normal expression

In a study examining geographical variation of *HLA-B*51* in BD, *HLA-B*51* was sequenced in 24 patients with BD and 13 healthy controls of Japanese, Turkish, Jordanian, and Iranian decent (Takemoto et al., 2008). The authors confirmed that all individuals carried *HLA-B*51:01:01* with no variation in the exons, introns, or 5'-flanking region. This study confirmed that the allele associated with BD is likely to be *HLA-B*51:01:01* (as opposed to a different subset of *HLA-B*51* alleles). The following year, a large meta-analysis examining the role of *HLA-B5* and *HLA-B*51* in BD was carried out. Eighty papers met the inclusion criteria for analysis. The authors found that in a pool of 4800 BD patients and 16, 289 controls from across the world, the pooled odds ratio (OR) was 5.78 (5.00-6.67 95% CI). Moreover, the effect was similar for patients from regions with a lower incidence of *HLA-B*51* in the control population, such as Northern Europe (Table 1-7) (de Menthon et al., 2009).

Over the years there have been numerous studies analysing the effect size of *HLA-B*51* in BD, ranging from 1.18 (0.34-4.07 95% CI) (O'Duffy et al., 1976) to 34.63 (4.13 – 290.22 95% CI) (Pittoni et al., 2003) (Table 1-7). Comparisons between these studies

should be drawn with care for a number of reasons; firstly, the sample populations and the control populations may overlap in different studies; secondly the techniques used for genotyping may vary and thirdly publication bias may skew the data (publication bias analysis in the de Menthon paper was equivocal and depended on the techniques used for analysis (de Menthon et al., 2009)).

Whether the *HLA-B*51* has a causative role in disease pathogenesis or is merely associated with the disease due to confounding factors has been a matter of speculation for decades. It is noteworthy that all 80 papers analysed by de Menthon et al. reported an odds ratio of > 1. Given the overall OR of 5.78, it is less likely that the association between *HLA-B*51* and BD is entirely due to confounders such as LD (Campbell et al., 2002).

In 2014, Ombrello et al. performed a large association study involving 1,190 BD cases and 1,257 matched controls from Turkey and an ethnically diverse European reference panel of 5,225 individuals. The study focused on single nucleotide polymorphisms (SNP) within the major histocompatibility complex (MHC) and used both direct typing and imputation based methods based on previous genome wide association (GWA) data (Ombrello, Kirino, et al., 2014). Using stepwise and multivariate logistic regression over HLA-B and adjusting for multiple SNPs revealed no additional genome-wide significant associations for markers in the HLA-B or the major histocompatibility complex class I chain related gene A (MICA) region.

Table 1-7. Pooled Odds Ratios For *HLA-B*51/B5* And Their Association With Behçet's Disease

*While HLA-B*51 has a robust effect on disease-risk, there is significant heterogeneity between study-populations across all ethnicities. Phet – P for heterogeneity statistic. n pop – number of populations studied. Adapted from (de Menthon et al., 2009)*

Pooled prevalence for HLA-B*51/B5						
Subgroups	**n Pop**	**BD (95% CI)**	**Control (95% CI)**	**OR (95% CI)**	**I^2 (%)**	***P*het**
Overall	80	57.2 (53.4-60.0)	18.1 (16.1-20.3)	5.78 (5.00-6.67)	60.6	0.0001
Eastern Asia	25	55.0 (49.8-60.1)	19.6 (16.0-23.7)	5.18 (4.15-6.47)	52.2	0.001
Middle East/North Africa	27	63.5 (58.8-68.0)	21.7 (18.2-25.7)	6.25 (4.87-8.03)	70.4	0.0001
Southern Europe	15	60.6 (51.9-68.7)	16.8 (13.3-21.0)	7.20 (4.89-10.62)	57.2	0.003
Northern /Eastern Europe	11	39.0 (28.2-51.1)	11.2 (8.1-15.3)	5.31 (3.35-8.40)	55.6	0.013
North America	2	34.2 (6.0-80.8)	18.0 7.6-37.1)	2.35 (0.56-9.82)	57.0	0.13

Two regions between *HLA-B/MICA* and *HLA-A/HLA-F* achieved genome-wide significance ($P<1.7\times10-8$). Of note, *HLA-B*51*, *B*15, B*27, B*57*, and each contributed independently to BD risk (Figure 1-4). Protective effects were seen from *HLA-A*03* and *HLA-B*49*.

One year prior to the publication by Ombrello et al., Hughes et al. reported contrasting findings from a Turkish discovery (503 cases and 504 controls) and Italian validation (144 cases and 1,270 controls) cohort (Figure 1-5). The authors used dense HLA typing and imputation to mine the region for associated SNPs. The results were subjected to a meta-analysis using control haplotypes from the 1,000 Genomes Project and the data revealed that the strongest association signal was a SNP, rs116799036, located approximately 24 kb upstream of HLA-B and 18 kb upstream of MICA with genome-wide significance.

Figure 1-4. Fine Mapping Of The HLA Locus In A Turkish Cohort

The authors used a European reference panel and found two regions achieving genome wide significance (P<1.7×10−8): the region between HLA-B/MICA and HLA-A/HLA-F. Reproduced with permission from (Ombrello, Kirino, et al., 2014).

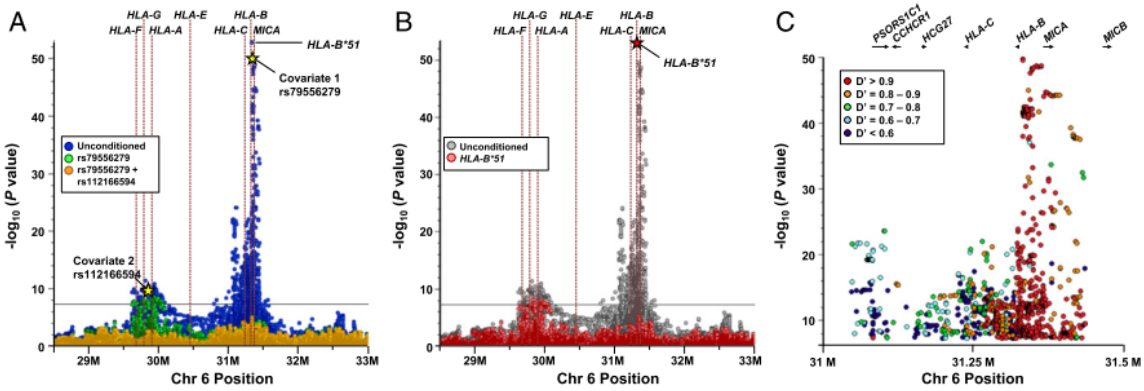


Fig. 1. *HLA-B*51* is the predominant risk allele, but variants between *HLA-F* and *HLA-A* are independently associated with BD. (*A* and *B*) The results of association testing and stepwise conditional analysis of imputed MHC region SNPs in 1,190 BD cases and 1,257 healthy control subjects from Turkey are displayed in *A*. Conditional analysis accounting for the effect of *HLA-B*51* (*B*, red dots) produced a pattern of residual association virtually identical to that seen after conditioning for rs79556279 (*A*, green dots). (*C*) Association testing results of BD-associated SNPs in proximity to *HLA-B/MICA* were plotted, and data points were color-coded to demonstrate D′ of each SNP with *HLA-B*51*.

Figure 1-5. Meta-Analysis Of 24,834 Markers In Turkish And Italian Cohorts

*The authors found that the effects of rs116799036 were significant despite controlling for HLA-B*51. When the effects of rs116799036 were controlled for, the association with HLA-B*51 were abrogated. Reproduced with permission from (E. H. Hughes et al., 2005)*

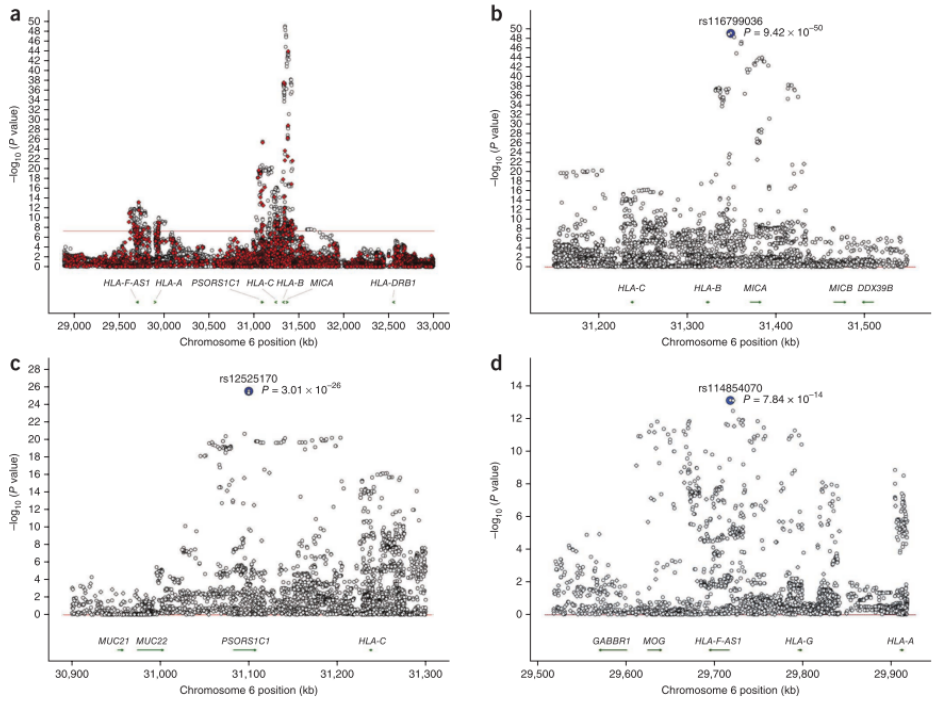


Figure 2 Meta-analysis results of 24,834 markers genotyped or imputed in the HLA region in both the Turkish and Italian cohorts included in this study. (**a**) Results from genotyped and imputed markers evaluated in the meta-analysis are depicted in red and black, respectively. The red line represents the genome-wide level of significance (5×10^{-8}). (**b**–**d**) Regional plots depicting meta-analysis results in the *HLA-B–MICA* locus (**b**), the *PSORS1C1–HLA-C* locus (**c**) and the *HLA-F-AS1–HLA-A* locus (**d**). Genotyped SNPs and imputed SNPs are depicted with diamonds and circles, respectively.

To determine whether the effect of rs116799036 could be explained by LD to *HLA-B*51:01*, and vice versa, Hughes et al. performed conditional analyses. Surprisingly, a genome-wide significant association of rs116799036 remained after conditioning on *HLA-B*51*:*01*. On the other hand, the genome-wide significant association of *HLA-B*51*:*01* completely disappeared after conditioning on rs116799036. These findings suggested *HLA-B*51* may not have a primary association with BD, but be associated via epistasis.

The association of rs116799036 identified by Hughes et al. was also identified by Ombrello et al. but the effect was seven orders of magnitude weaker than that of *HLA-B*51*. Furthermore, the association of *HLA-B*51* with BD remained significant even after conditioning for the effect of rs116799036.

Various theories have been suggested to explain the role of *HLA-B*51* in disease pathogenesis. HLA class I molecules play a central role in the immune system and, despite the lack of direct experimental evidence for this theory, it is hypothesised that *HLA-B*51* triggers BD by means of selective binding of pathogenic peptides (Verity et al., 1999).

In favour of a causative hypothesis is the strong association between the disease and presence of *HLA-B*51*:*01:01* in different populations (Takemoto et al., 2008). Dense genotyping arrays using large discovery and validation cohorts have found a similar association (Kappen et al., 2015; Ombrello, Kirino, et al., 2014). Furthermore, it seems logical that an error in antigen presentation may cause an inflammatory disease such as BD. However, this argument does not explain the pathogenesis in *HLA-B*51*- individuals, nor does it propose a pathogenic pathway that could be investigated further.

1.4.3 The Role of *HLA-B*51* in Behçet's Disease

*HLA-B*51*'s role in the development of BD has yet to be clarified. Available data suggests that there is no single pathogenic mechanism associated with *HLA-B*51*, and its effects may be mediated by a combination of different HLA Class-I associated functions and structural properties.

*1.4.3.1 Presentation of HLA-B*51-Restricted Peptides to CD8 T Cells*

One possible role may be the presentation of HLA-B*51-restricted peptides to $CD8^+$ T cells inducing a disease-specific adaptive immune response. A key characteristic of HLA-B*51 is its ability to bind a large pool of peptides including those with low-affinity, thus allowing promiscuous behavior in the presentation of peptides to $CD8^+$ T cells (Gebreselassie et al., 2006).

It has also been reported that there are increased proportions of $CD8^+$ T cells and $\gamma\delta^+$ T cells in patients with active disease, especially those who possess HLA-B*51, compared toinactive patients and healthy controls (Yasuoka et al., 2008).

However, to date, there have been no in vivo studies that have shown HLA-B*51 restricted $CD8^+$ T cell activation or disease-specific pathogenic cytotoxic immune responses.

*1.4.3.2 Structural and Ligand-Binding Properties of HLA-B*51*

The structure of HLA-B*51 molecule may itself trigger an autoinflammatory response. The unique folding and ligand binding properties of HLA-B*51 molecule distinguish it as a slow-folding MHC molecule (Giza et al., 2018; Sakaguchi et al., 1997). Low affinity peptides can lead to slow assembly and misfolding of the HLA-B*51 molecule, which could lead to an autoinflammatory response and stress within the endoplasmic reticulum (ER). The ER stress and unfolded protein response have been proposed as possible pathogenic mechanisms associated with *HLA-B*27* in ankylosing spondylitis, another MHC Class I-associated disease, based on data coming from *HLA-B*27*–transgenic animal models (Turner et al., 2007). But, there is as yet no evidence supporting the HLA-B*51-associated ER stress in the pathogenesis of BD.

*1.4.3.3 Presentation of HLA-B*51 Derived Peptides by HLA Class II*

It is possible that peptides derived from the digestion of HLA-B proteins, including HLA-B*51, can be presented by HLA Class-II molecules and induce an adaptive immune response.

There have been previous reports that a polymorphic amino acid sequence derived from digestion of HLA-B*27 shared homology with retinal S-antigen, an antigen associated with uveitis (Wildner et al., 1994). While it is intriguing to hypothesise that autoinflammatory responses could be generated from breakdown of specific HLA Class I molecules, this observation cannot explain the specific association of *HLA-B*51* allele with BD.

*1.4.3.4 Increased Neutrophil Activation In HLA-B*51^{+} Transgenic Mice*

There is no established animal model for BD. Historically, there have been a number of attempts to create such an animal. The herpes simplex virus (HSV) mouse model has been suggested as a candidate, as some animals get recurrent mucosal ulceration and inflammatory bowel and joint disease. (Cho et al., 2013; Choi, Kim, et al., 2011). There is no established HLA-B*51 transgenic animal model for testing all possible pathogenic mechanisms related to HLA-B*51in BD pathogenesis. One group in Japan, developed an HLA-B*51^{+} transgenic mouse model, which developed no symptoms of BD, but did display increased neutrophil activation, compared to HLA-B*35 and wild-type mice (Takeno et al., 1995). This observation has not been repeated or fully explained by the inclusion of HLA-B*51 into the system, however similar enhanced neutrophil activity has been observed in reported in HLA-B*51^{+} healthy individuals as well as in BD (Sensi et al., 1991; Takeno et al., 1995).

1.4.4 Genome Wide-Association Studies

To date, eight genome wide association studies (GWAS) investigating BD have been carried out and a number of candidate genes have been implicated as well as non-coding regions located between the HLA-A - HLA-F loci (Meguro et al., 2010; Mizuki et al., 2010). These GWAS studies have mainly been carried out in the regions of the world where BD is most prevalent (Turkey, Japan, China, Korea and Iran), although recently a mixed population of patients with BD from the Netherlands was reported (Fei et al., 2009; Hou et al., 2012; Kappen et al., 2015; Kirino et al., 2013; Y. J. Lee et al., 2013; Mizuki et al., 2010; Remmers et al., 2010; Xavier et al., 2015). All GWAS studies reported an

association within the MHC, but there has been considerable inter-study heterogeneity with regard to other reported associations. The key associations are reported in Table 1-8.

Table 1-8. Other Single Nucleotide Polymorphisms Associated With Behçet's Disease

There have been eight genome wide association studies in BD. While some of the findings have been replicated across different populations, others have only been found in specific study populations. PBMCs – peripheral blood mononuclear cells, LPS – lipopolysaccharide, MDP – muramyl dipeptide. Gene acronyms can be found in the glossary. Adapted from Takeuchi et al, 2015. (Takeuchi et al., 2015).

Variant	**Gene**	**Location**	**Risk Allele**	**OR**	**Population**		**Function**
					Discovery	**Validation**	
rs1495965	IL23R, IL12RB2	Intergenic	G	1.35	Japanese	Turkish	
rs924080	IL23R, IL12RB2	Intergenic	A	1.28	Turkish and Japanese		
rs1518111	IL10	Intron	A	1.45	Turkish	Greek, UK, Iranian, Middle Eastern, Arab, Japanese, Han Chinese	Reduces expression in monocytes
rs1800871	IL10	Promoter	T	1.45	Japanese	Turkish, Korean, Han Chinese	
rs9494885	TNFAIP3	Intergenic	C	1.81	Han Chinese		No difference in expression in PBMCs
rs7574070	STAT4	Intron	A	1.27	Turkish and Japanese		Increases expression
rs897200	STAT4	Intergenic	A	1.45	Han Chinese		Increases expression of STAT4 and IL17
rs7616215	CCR1	Intergenic	T	1.39	Turkish	Japanese	Decreases expression in monocytes and reduces monocyte chemotaxis

rs13092160	CCR1, CCR3	Intergenic	T	3.13	Han Chinese		Decreases expression in PBMCs
rs2617170	KLRC4	Missense	C	1.28	Turkish and Japanese		Tag for haplotype associated with increased cytotoxicity of PBMCs
M694V	MEFV	Missense	V	2.65	Turkish		Increases response to LPS
rs17482078	ERAP1	Missense	TT	4.65	Turkish		Tag for haplotype with reduced peptide trimming activity
rs681343	FUT2	Synonymous	T	1.30	Iranian and Turkish		r^2=1 with a nonsecretor allele (rs601338)
rs1781046	IL12A	Intergenic	A/G	1.66	Turkish and mixed population		
R381Q, G149R	IL23R	Missense, missense		Protective	Japanese and Turkish		Reduces IL-23 dependent IL-17 (R381Q)
D299G, T399I	TLR4	Missense, missense		Protective	Japanese and Turkish		Reduces reponse to LPS
R702W, G908R, L1007fs	NOD2	Missense, missense, frameshift		Protective	Japanese and Turkish		Reduces response to MDP

1.4.5 The Major Histocompatibility Complex Class I Chain Related Gene A (MICA) - Nomenclature

As previously mentioned, the international ImMunoGeneTics information system (IMGT) has created specific nomenclature for genes and proteins involved in the immune response. The nomenclature for *MICA* alleles follows the standardised rules of the IMGT (Lefranc et al., 2005). *MICA* sequences are defined for each allele the following IMGT criteria:

1. First sequence published
2. Longest sequence
3. Mapped sequence

IMGT allele names are identified by the gene name followed by an asterisk and a 3-digit number, i.e. *MICA*001.*

1.4.6 *MICA* Transmembrane Polymorphisms

In 1997, Mizuki et al. found that a polymorphic microsatellite or Short Tandem Repeat (STR) was found in exon 5 (Mizuki, Inoko, et al., 1997). The STR consists of a variable number of trinucleotide GCTs encoding 4, 5, 6 or 9 Alanine (A) repeats and were accordingly named as A4, A5, A6, A9 and A10. There is also an A5.1 allele that contains five triplet repeats plus one additional nucleotide 'G'. This insertion leads to a frameshift and results in a stop codon and premature termination. A substantial body of literature exists describing these STRs and their disease associations. A paper may only refer to the STR when describing the allele. This thesis will follow World Health Organisation (WHO) guidelines (Frigoul et al., 2005) and refer to an allele by its IMGT nomenclature followed by the STR in parenthesis, i.e. *MICA*009* (A6).

1.4.7 *MICA* Background

The *MIC* locus is located approximately 46kb from *HLA-B* between *HLA-B* and *TNFα* (Bahram et al., 1994). The *MICA* transcript of 1482bp encodes a 383 amino acid polypeptide of 43kDa that is structurally very similar to classical MHC class I molecules. It is composed of 3 extracellular domains referred to as α1 (encoded by exon 2), α2

(encoded by exon 3) and α3 (encoded by exon 4), a transmembrane region (encoded by exon 5) and a carboxy-terminal cytoplasmic tail (encoded by exon 6) (Bahram, 2000). However, it does not carry peptide cargo; is not complexed to β2-microgloubulin (B2M) and does not associate with transporter associated with antigen processing (Groh et al., 2002). It is closely related to a sister protein, MICB, encoded from the same locus.

Both MICA and MICB are stress inducible ligands for a C-type lectin receptor, natural killer group 2, member D (NKG2D), expressed primarily by subsets of cytolytic $CD8^+$ T cells, γδ T cells, and NK cells (Bauer et al., 1999). MICA proteins are expressed on primary tumours of epithelial origin (Dayanc et al., 2013; Ghadially et al., 2017) on leukaemias (N. Kato et al., 2007; Weiss-Steider et al., 2011); and on virus-infected cells (Goto et al., 2015; Tong et al., 2013). Recognition of MICA ligands by NKG2D homodimer receptors induce cytotoxic activity of NK cells and subsequent lysis of tumour or virally infected cells (Weiss-Steider et al., 2011).

MICA is recognised as the most polymorphic, human, non-classical class I gene, with at least 87 alleles (http://hla.alleles.org/data/mica.html). Several studies have shown *MICA*008* (A5.1) to be the most frequent allele in a variety of populations, with an allele frequency up to 74% in Caucasian populations (Fodil et al., 1999; Lucas et al., 2008; Luo et al., 2014; Norris et al., 2001; W. Tian et al., 2006). *MICA*002* (A9) and *MICA*004* (A6) are more frequent in Sub-Saharan Africa (W. Tian et al., 2003) and *MICA*020* and **027* are most frequent amongst South American Indians (Oliveira et al., 2008).

1.4.8 *MICA* In Behçet's Disease

Extensive work has been carried out over the past 2 decades to investigate the effect of *MICA* on BD pathogenesis and heritability (Frigoul et al., 2005). *MICA*009* (A6) has been implicated in BD in Southern European, Middle Eastern, Central Asian and East Asian populations (Table 1-9). The majority of studies have found that the *MICA*009* (A6) effect is abrogated when controlling for *HLA-B*51* as the two genes are in strong LD with each other. However, there is evidence to suggest that possession of *HLA-B*51* and A6 may have a cumulative effect increasing the risk of developing BD and, in Korean patients, it may carry the majority of the susceptibility (Park et al., 2002). It has also been suggested that the *HLA-B*51*/A6 effect may be largely mediated by a SNP at rs76546355 (Carapito et al., 2015).

A meta-analysis investigating the effects of *MICA* polymorphisms in BD was carried out in 2014 (Y. H. Lee et al., 2015). The authors found that *MICA* A6 and *MICA*009* were both significantly associated with BD in all populations (OR 1.653, 95% CI 1.409-1.937, P<1.0x10-8), in Europeans (OR 1.436, 95% CI 1.111-1.857, P=0.006) and in Asians (OR 1.999, 95% CI 1.551-2.575, P=8.0x10-8), but not in the Iranian, Arabic or Jewish populations. As expected, the association was significantly stronger for BD patients who also carried the *HLA-B*51* gene. *MICA*009* was also significantly associated with BD overall (OR 3.948, 95% CI 2.680–5.815, P<1.0x10−8); in Europeans (OR 3.392, 95% CI 2.118-5.433, P=5.6x10-6) and in a Turkish population (OR 5.417, 95% CI 2.744-10.69, P=1.1x10-7). The authors found that *MICA**009 was also strongly associated with *HLA-B*51*$^{+}$ individuals, but not *HLA-B*51*$^{-}$ individuals.

Table 1-9. *MICA* Allele And Microsatellite Repeat Associations In Behçet's Disease

NS - No allele or microsatellite found to be significant.

MICA Allele	**MICA Exon 5 TM microsatellite repeat**	**Ethnicity**	**Significant effect after Controlling for *HLA-B*51***	**Reference**
*MICA*009*	A6	East Asian	Yes	(Mizuki, Ota, et al., 1997; Park et al., 2002)
NS	A6	Southern European	No	(Picco et al., 2002)
*MICA*009*	A6	Middle Eastern	No	(Carapito et al., 2015; Wallace et al., 1999)
NS	A5/A6	Central Asian	No	(Y. L. Chung et al., 2003)
NS	NS	East Asian	No	(Mizuki et al., 1999; Mizuki et al., 2000)
NS	NS	Southern European	No	(Gonzalez-Escribano et al., 1999; Mizuki et al., 2000; Salvarani et al., 2001; Yabuki et al., 1999)
NS	NS	Middle Eastern	No	(Cohen et al., 2002; Mizuki et al., 2001)
NS	NS	North African	No	(Ben Ahmed et al., 2003)

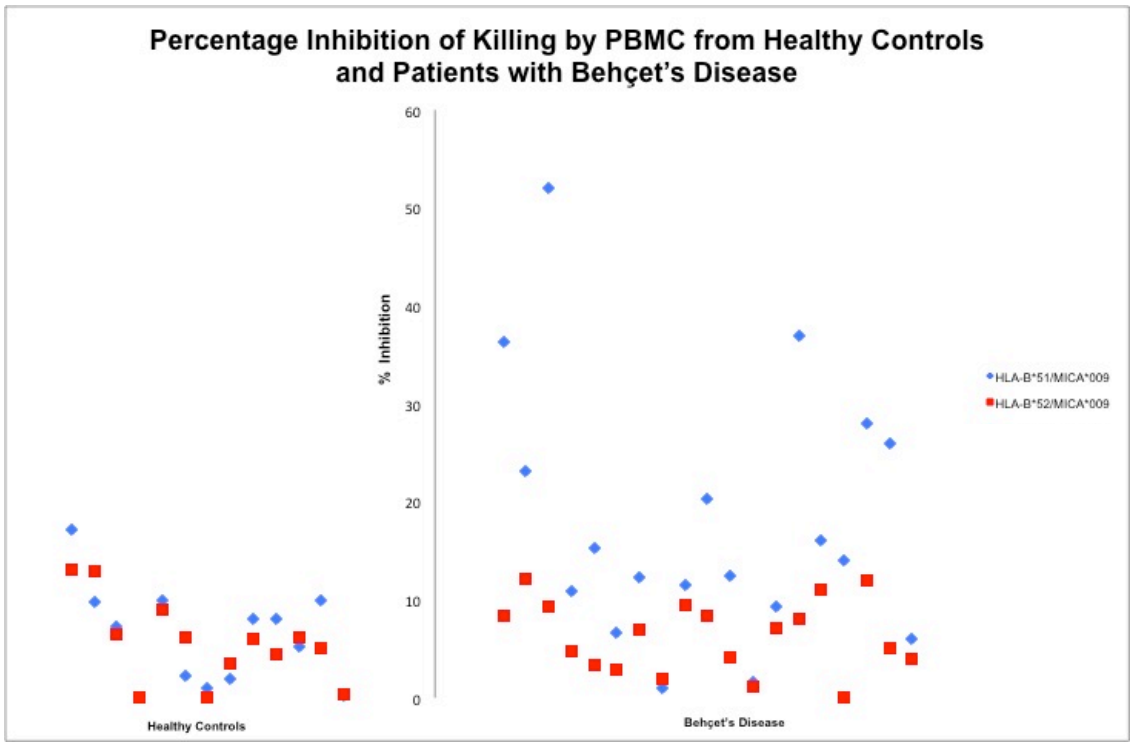
The association of *MICA* and MHC class I haplotypes with BD, suggests a major role in the disease for cells regulated by these molecules including NK cells and subsets of T cells (Mizuki, Ota, et al., 1997; Yabuki et al., 1999). While it is generally agreed that any association is likely to be due to linkage disequilibrium (LD) with *HLA-B*51* (Cambra et al., 2009; Gao et al., 2006; W. Tian et al., 2001), MICA may have a functional role independent of *HLA-B*51*. Wallace et al. demonstrated that while *MICA* was in LD with *HLA-B*51* and *HLA-B*52*, it only co-segregates with *HLA-B*51* in the BD population (Wallace et al., 1999).

The affinity of MICA as a ligand for NKG2D is not uniform. Low affinity MICA have a valine at codon 129 and include MICA*019 and MICA*009, these receptors have a 10-50 fold weaker affinity for NKG2D compared to those with methionine in position 129. Munoz-Saa et al. noted that none of their 42 BD patients expressed a high affinity phenotype for NKG2D compared to 14% of controls. Furthermore, autoimmune diseases such as anterior uveitis, ankylosing spondylitis and psoriatic arthritis are all associated with high affinity alleles. (Amroun et al., 2005; Gonzalez et al., 2001; Ricci-Vitiani et al., 2000)

Shafi et al. utilised a functional genomics system with a Chinese hamster ovary (CHO) cell line transfected with *HLA-B*51*/52 and MICA*008/*009/*027 to examine the role of MICA polymorphisms on NK and γδT cells. The investigators found that while certain alleles were more highly expressed, expression did not directly correlate with cytotoxicity. The group also identified that peripheral blood mononuclear cells (PBMC) from patients with BD produced a varied response to *HLA-B*51*/MICA*009 combinations, but the response to HLA-B*52/MICA*009 was uniform. Moreover, peripheral blood mononuclear cells (PBMC) from healthy controls showed uniform responses for both combinations. This suggests that other molecules involved in the cytotoxic response may be responsible for the differences seen (Figure 1-6).

Figure 1-6. Percentage Inhibition Of Killing By Peripheral Blood Mononuclear Cells From Healthy Controls And Patients With Behçet's Disease

*Healthy controls (left) showed a uniform response to target cell killing, whereas PBMC from HLA-B*51+ patients with BD (right) had a variable ability to kill. The reason for this difference in cytotoxicity levels was not fully explained by varying MICA/HLA alleles alone. Personal communication (Shafi et al, unpublished data).*



1.5 Infectious Aetiologies

When Hulusi Behçet first described the condition in 1937, he believed that BD was caused by a viral agent as he noted 'inclusion-like' bodies on smears from the anterior chamber of eyes with hypopyons and mucosal ulcers (Behçet, 1937). Since then, there have been a number of attempts to elucidate which, if any infectious agent may cause BD or contribute toward it.

1.5.1 Viruses

In 1953, Sezer et al. inoculated fluid from the eyes and aphthae of patients with BD into the chorioallotoic membrane of fertile chick-eggs and found inclusion-bodies similar to those described by Behçet in 1937. These inclusion bodies were then transferred into the brains of wild-type mice, which subsequently developed BD-like symptoms and died. (Sezer, 1953a, 1953b). Later, Eglin et al. used in situ hybridisation to identify HSV-1 from mucosal lesions of patients with BD (Eglin et al., 1982), which lead a number of groups to start measuring the presence of HSV antibodies and DNA in blood (Bang et al., 2008; Choi, Lee, et al., 2011; Hamza et al., 1990; Hamza et al., 1991; Hamzaoui et al., 1992; Kim do et al., 2013), as well as saliva (Kim do et al., 2013; S. Lee et al., 1996).

Other herpes viruses have also been investigated in BD. Tojo et al. measured the relationship between HSV-1 and 2 as well as the human herpes virus 6 (HHV-6) and 7 in tissue samples from patients with BD and other related inflammatory disorders. Four cases were positive for HSV-1; 1 of 11 BD cases, 2 of 3 neutrophilic dermatosis cases and 1 of 3 idiopathic erythema nodosum cases. The authors concluded that there might be some relationship between BD and the presence of HSV-1 and/or HSV-2 DNA and that HHV-6 and HHV-7 do not seem to be involved in disease pathogenesis (Tojo et al., 2003).

More recently, proteomic analysis has been used to detect target HSV protein as well as serum IgA and IgG reactivities in BD patients and BD-like mice (Zheng et al., 2015). Compared with controls, patients with BD and BD-like mice exhibited higher titres of IgA reacting with recombinant HSV protein. Further proteomics analysis revealed that human heat shock protein 71 (HSP71) cross-reacted with target antigen against anti-HSV antibodies. The authors suggested that HSV infection and impaired human HSP71 activity

may be associated with the activation of autoreactive lymphocytes, rather than HSV directly being the cause of BD.

Serum anti-HSV-1 antibodies are found in a higher proportion of patients with BD than in controls and circulating immune complexes including the HSV-1 antigen have been reported in BD patients (Lehner, 1997). When the data is compelling, many of these finding are replicated in HC and at present these findings have not been adopted into clinical practice and patients with BD are not routinely treated with antiviral agents to manage their disease. Similarly, several other viral agents, including hepatitis C virus, parvovirus B19, cytomegalovirus, Epstein-Barr virus, and varicella zoster virus, have also been described as associated with BD, although their role in disease pathogenesis remains unclear (Akdeniz et al., 2003; Ilter et al., 2000; Kiraz et al., 2001; Seoudi et al., 2015; Sun et al., 1998; Sun et al., 1996).

1.5.2 Bacteria and Bacterial Superantigens

Oral aphthae are frequently the first manifestation of BD (Mumcu et al., 2009; Seoudi et al., 2015) oral microbial flora have long been implicated in the pathogenesis of disease. Clinical observations such as increased oral manifestations after dental treatment, hypersensitivity to streptococcal skin tests, overgrowth of atypical streptococci in patients with BD and recent reports of beneficial antibacterial treatment suggest a role for streptococci in BD (Japan, 1989; Lehner, 1997).

In 1992, Hirohata et al. investigated KTH-1, an extract of Streptococcus sanguis SSH-83 that causes increased IL-6 and IFN gamma secretion by CD8 T cells in patients with BD (Hirohata et al., 1992). KTH-1 also up regulates gamma delta T cells in short-term T cell

cultures, and KTH-1- specific gamma delta T cell lines secrete the proinflammatory mediators IL6, IL8, and TNFα (Mochizuki et al., 1994). In addition to streptococcal antigens, Hirohata et al. described the ability of Escherichia coli and Staphylococcus aureus to activate lymphocytes and release increased amounts of IFN α and IL-6 in BD. The same group found that CD8 T cells from BD patients require lower doses of the staphylococcal superantigens SEB and SEC1 to secrete IFN α, leading to the hypothesis that there is a degree of T cell hyperreactivity in BD (Hirohata et al., 1998).

1.6 Heat Shock Proteins

The 60 and 65 kilodalton (kDa) heat shock proteins (HSP) have also been thought to contribute toward the development of BD. HSPs are proteins expressed by cells undergoing stress. Initially, this was described after exposure to heat, but subsequently it has been demonstrated that HSPs are upregulated after a number of stress-inducing conditions in order to stabilise the surface expression of other cell-signalling receptors (Whitley et al., 1999). There is substantial homology (>70%) between mammalian and microbial HSP, leading to the hypothesis that CD8 T cells responsive to bacterial HSP may provoke an autoinflammatory response in humans via immunological adjuvantcy (Kaufmann et al., 1991).

HSP65 is expressed abundantly in epidermal regions of active skin lesions, such as erythema nodosum and mucocutaneous ulcers in BD (Ergun et al., 2001). In 1991, Lehner et al. suggested that HSP65 may function as a pathogenic antigen in BD by identifying anti-HSP65 antibodies that are cross-reactive with oral mucosal homogenates and oral streptococci (Lehner et al., 1991). Additional HSP derivatives were found to lead to increased activation in BD patients. T cell responses to mycobacterial HSP65-derived

peptides and to their 50–80% homologues on human HSP60 were shown to be significantly higher in patients with BD from UK, Japan, and Turkey than in controls (Direskeneli et al., 2000; S. Kaneko et al., 1997).

Purified protein derivative and HSP65-specific long-term T cell lines are also highly reactive to human HSP60-derived peptides in both patients with BD and HC. Most purified protein derivative-stimulated lines responded to a specific epitope 425–41 of HSP60 in patients with BD, whereas epitope 336–51 dominated in controls, suggesting that the patterns of response and the immunodominance of epitopes may differ in patients with BD from those of HCs (Direskeneli et al., 2000).

Cross reactivity is also demonstrated for anti-HSP60 antibodies. Both antistreptococcal and antiretinal HSP60 antibodies are raised in the serum samples of patients with BD and uveitis, with significant cross-inhibition (Tanaka et al., 1999). Increased anti- HSP65 antibody responses are also present in the cerebrospinal fluid (CSF) of patients with neuro-BD with parenchymal involvement (Tasci et al., 1998).

1.7 Innate Immunity - A Role For Natural Killer Cells In Behçet's Disease

Natural killer (NK) cells are lymphocytes of the innate immune system. They are involved in the early stages of defence against foreign and self-cells undergoing stress. When stimulated, NK cells secrete an array of cytokines including interferon gamma (INFγ), tumor necrosis factor α, (TNFα), granulocyte-macrophage colony-stimulating factor (GM-CSF), and chemokines such as chemokine ligand 3 (CCL3), macrophage inflammatory protein 1 α (MIP1α) and CCL4 (MIP1β) (Dorner et al., 2002). NK cells are also cytotoxic, inducing apoptosis of target cells. NK cells identify their targets

through a set of activating or inhibitory receptors, which recognise non-self-targets as well as self-proteins that are upregulated as a result of stress. They can also kill cells that down-regulate expression of self-MHC molecules during times of infection or transformation (Raulet, 2004; Walzer et al., 2005). In both viral and bacterial animal models of infection, IFNγ production by NK cells has been identified as the key event for successful pathogen eradication (Byrne et al., 2004), which is driven by early IL-12 production (Bancroft, 1993; Orange et al., 1996).

Natural killer cells comprise 10-15% of circulating lymphocytes in humans, falling mostly into two distinct populations. Approximately 90% are $CD56^{Dim}CD16^{+}$ cells, capable of killing and antibody dependent cytotoxicity. These cells produce relatively little IFNγ (Caligiuri, 2008; Inngjerdingen et al., 2011). The other 10% are $CD56^{Bright}$CD16- and are weakly cytotoxic. These cells do not proliferate vigorously in response to IL-2, but produce substantial amounts of IFNγ (Cooper et al., 2001). It has been suggested that $CD56^{Bright}$CD16- cells utilise their cytokine production to play a regulatory role in immune responses while $CD56^{dim}CD16^{+}$ cells are terminally differentiated cytotoxic effector cells (De Maria et al., 2011).

Natural killer cells are decreased in patients with autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, Sjogren's syndrome, rheumatoid arthritis (RA) and type I diabetes (French et al., 2004; Z. Tian et al., 2012) and have been found to be increased in ankylosing spondylitis, a condition associated with HLA-B*27, which also expressed the Bw4 epitope (Azuz-Lieberman et al., 2005). However, their role in autoinflammatory diseases such as BD is less clear, with conflicting evidence of NK cell involvement (Hamzaoui et al., 1988; F. Kaneko et al., 1985; Suzuki et al., 1992).

A complex balance of activating and inhibiting surface receptors control NK cells. Furthermore, depending on their location, NK cells can express variegated levels of activating and inhibiting receptors tailored to their environment.

1.7.1 Natural Killer Cell Licensing

Natural killer cells are 'licensed' to recognize and kill cells that do not express self-MHC. In 1986 Karre et al. described this phenomenon as the 'missing self' hypothesis (Karre et al., 1986). This states that NK cells are activated to detect and kill cells that are thought to be non-self, such as infected cells or those undergoing neoplastic changes. This theory was not able to explain the finding that NK cells from MHC-deficient mice and humans do not effectively kill target cells (Furukawa et al., 1999). Discovery of the killer immunoglobulin-like receptor (KIR) gene complex and the role the role of KIR as a ligand for HLA has added significantly to the understanding of NK cell biology and licencing.

In any one individual, some NK cells do not have a corresponding inhibitory HLA ligand i.e. KIR3DL1^{+} cells in a Bw6 homozygote. It is unclear how such cells could be prevented from killing autologous cells. Yokoyama et al. proposed that KIR on NK cells must recognize their cognate HLA ligand in order to acquire functional competence through licensing (K. Kato et al., 2006). An alternate 'disarming' hypothesis proposes that NK cells that fail to recognise MHC class I via inhibitory KIR become anergic (Raulet, 2006). Hence, paradoxically, an inhibitory receptor interaction appears to be required for an NK cell to acquire function. In the context of infection, unlicensed NK cells may become activated in response to cytokines from infected cells. This may permit a broader repertoire of NK cells to contribute to the response to pathogens without the risk of autoimmunity (Kimura et al., 2005). Moreover, the licensing concept embraces gene

dosage, i.e. KIR3DL1^{+} cells in donors who are homozygous for Bw4 display increased responsiveness to tumour stimulation compared to heterozygotes and those who lack the ligand. By contrast NK cells lacking KIR3DL1 show no difference in activity (Boulet et al., 2008).

1.7.2 The Killer Immunoglobulin-Like Receptor 3DL1/S1 (KIR3DL1/S1)

HLA-B*51 presents antigen to CD8^{+} cytotoxic T cells, but is also known to interact with the killer immunoglobulin-like receptor 3DL1 (KIR3DL1). KIR are found on NK, T and γδT cells and interact with HLA-B*51 via its Bw4 epitope (Gumperz et al., 1995). Positions 7 and 8 of the KIR3DL1 peptide interact at positions 77, 80, 81, 82, and 83, which form the HLA-Bw4/Bw6 epitope (Peruzzi et al., 1996). Bw4 is present on a third of all HLA-B types and is defined by leucine at position 82 and arginine at position 83. It is associated with strong inhibition of NK cell activity via KIR3DL1 receptor interaction. The Bw6 epitope (Ser 77, Asn 80, Leu 81, Arg 82, and Gly 83) does not interact with KIR3DL1 (Parham et al., 2011). The Bw4 epitope can be found on numerous other HLA types (http://hla.alleles.org/antigens/HLA-Bw46.html) some of which are also associated with BD, such as *HLA-B*27*:02 (Gul et al., 2002).

In humans, NK receptors belong to either the immunoglobulin-like superfamily (IgSF) or the C-type lectin-like receptor (CTLR) superfamily. KIR are part of the IgSF and have specific ligands in the HLA class I family (Lanier, 1998). Inhibitory KIR (i.e., KIR2DL and KIR3DL), contain immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic domains. Activating KIR molecules (i.e., KIR2DS and KIR3DS) lack ITIM and have a charged residue in their transmembrane domains, which likely pair with the DAP12 signalling adapter. KIR2DL4 is unusual as it consists of a long intracellular region

but only one ITIM and a positively charged amino acid in the transmembrane region. Unlike other clonally distributed KIR, KIR2DL4 is transcribed by all NK cells and acts as an activating receptor on recognition of HLA-G (Faure et al., 2002; Rajagopalan et al., 2012). Inhibitory KIR consistently binds with a higher affinity than their activating counterparts (Katz et al., 2001).

1.7.2.1 KIR3DL/S1 Allelic Divergence

Ancestral linages of *KIR3DL/S1* alleles have been extensively investigated by Parham et al. over the past decade. Evidence has arisen to suggest that the polymorphic nature of the *KIR* gene cluster has, in part, developed as a result of changing cognate HLA-ligands (Parham et al., 2011). As part of this development, *KIR* genes have expanded from one common ancestral *KIR – KIR3DX* around 120 million years ago (Guethlein et al., 2015). At this time, placental mammals expanded their KIR3DL lineages to varying degrees. Figure 1-7 describes the evolution of *KIR3DL/S1* in humans. As a result of this extensive allelic divergence, KIR3DL1 allotypes have variegated expression as can be seen in Figure 1-8. KIR3DL1*005 and *007 are low-expressing ($KIR3DL1^{LOW}$) allotypes but evolved from different ancestral *KIR3DL1* alleles (Figure 1-7). The reason for two alleles having similar function, but different ancestry is unclear, but suggests that both high and low-expressing KIR3DL1 allotypes are required for a robust, but controlled innate immune response.

Figure 1-7 Phylogenetic Relationships And Geographic Distribution Of KIR3DL1/S1 Alleles

*Three divergent 3DL1/S1 allelic lineages are maintained in all human populations. The distance between two nodes corresponds to one nucleotide change in the coding region.. Nodes with coloured circles are the alleles present in the modern human population, the area representing the frequency worldwide and the different colours the distribution between major population groups. Allelic lineages are denoted by the background shading: *015, magenta; *005, cyan; and 3DS1, green. 3DL1*001, a recombinant of the *015 and *005 lineages, has a purple background. Dashed lines indicate four other recombination events: R1, acquisition of activating signalling function to form 3DS1 from 3DL1 (the 22 unique substitutions in the 3DS1 signalling domain are not shown as nodes, because they were acquired en bloc); R2, causing 3DL1*007 and 3DL1*004-like alleles to have the same cytoplasmic tail; R3, forming a chimera of 3DL1 and 3DL2; and R4, representing two independent events when 3DL1 acquired the D0 domain of 3DS1 to give the 3DL1*009 and 3DL1*042 alleles – Reproduced with permission from (Parham et al., 2011).*

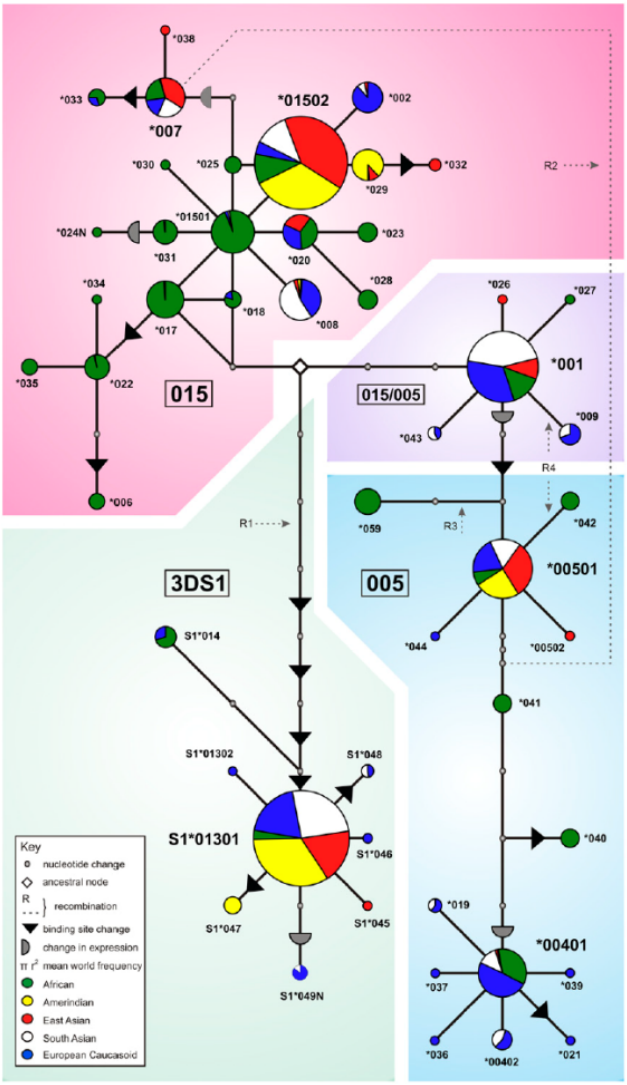
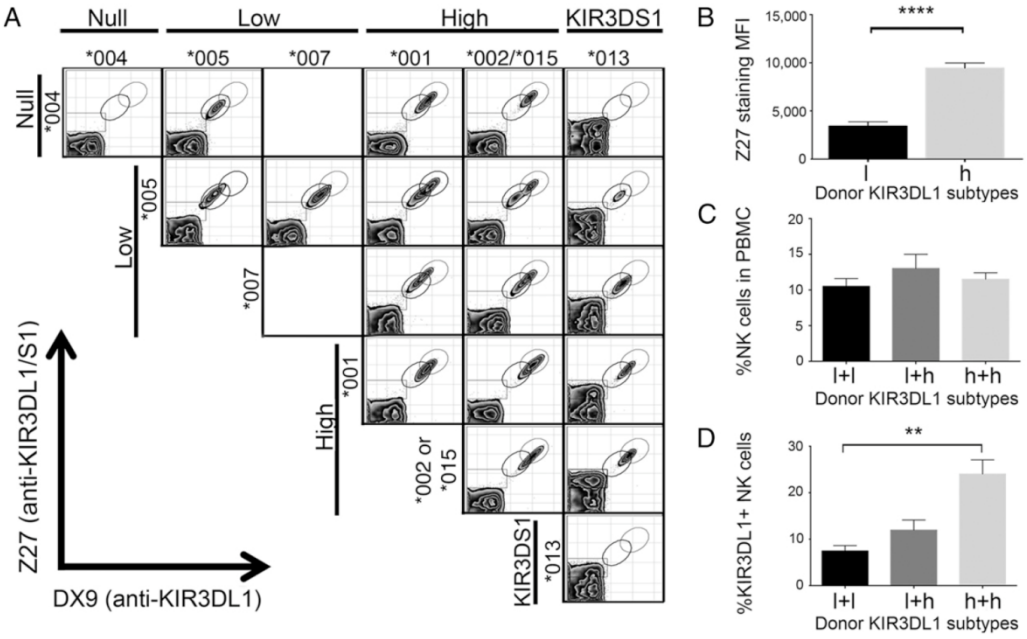


Figure 1-8. KIR3DL1 Allotypes Allow Predictable Binding Patterns To Be Observed In Healthy Controls.

*Viable CD3^{-}CD56+ NK cells from healthy human donors were assessed by KIR3DL1 PCR-SSP and stained for flow cytometry using DX9 (anti-KIR3DL1) and Z27 (anti-KIR3DL1/S1) mAbs. (A) Phenotyping panel of healthy donor PBMC, gated on NK cells to represent all available major allele subtype combinations. Labels indicate the most common allele in each KIR3DL1 subtype. A representative donor from each subtype combination is displayed. (B) Z27 staining MFI from individuals expressing KIR3DL1^{LOW} (l) or KIR3DL1^{HIGH} (h) (C) Percentage of PBMC that are NK cells, segregated based on donor compound KIR3DL1 subtypes. (D) Percentage of peripheral blood NK cells that express KIR3DL1, organized based on donor KIR3DL1 subtypes. Bar graphs represent means 6 SEM and a minimum of two independent experiments with three to five donors per group. Means are compared by student t test (B) or one-way ANOVA (C and D). **P<0.01, ****P< 0.0001. – Reproduced with permission from (Boudreau et al., 2016). KIR – Killer Immunoglobulin-like Receptor, PBMC – Peripheral Blood Mononuclear Cells, ANOVA – Analysis of Variance*



1.7.3 KIR3DL1 Expression In Behçet's Disease

Saruhan-Direskeneli et al. found no association of KIR3DL1 expression on NK or T cells in patients with BD, a finding not altered by the presence of Bw4 motif in patients (Saruhan-Direskeneli et al., 2004). Similar results were found by Middleton et al. after analysing 14 *KIR* genes in a cohort of Turkish patients and ethnically matched controls using a sequence-specific oligonucleotide probe (SSOP) method. They reported an increased frequency of *KIR3DL1* and its ligand Bw4 (P=0.0003) and a corresponding decreased frequency of *KIR3DL1* without Bw4 (P=0.00004) in patients compared to controls. However, this difference was abrogated when the presence of *HLA-B*51* was controlled for (P=0.7075) (Middleton et al., 2007).

The frequency of 16 KIR genes (2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DS1, 2DP1 and 3DP1) was assessed in Korean patients with either *HLA-B*27*-associated ankylosing spondylitis and uveitis or BD related uveitis. In the patients with ankylosing spondylitis, the frequency of KIR3DL1 was significantly lower than healthy controls (P=0.043), while there was no difference in patients with BD (Moon et al., 2013). These studies support the evidence that KIR3DL1 interacts with HLA-B*51 via Bw4; however, there is currently insufficient evidence to suggest that KIR3DL1 or its subtypes have a role in the pathogenesis of BD. The KIR cluster is highly polymorphic, second only to the HLA in complexity and is an unsuitable candidate for GWAS analysis as SNP probes targeting the region are unable to bind due to allelic and copy number variation within the gene. Other methods including targeted sequencing and functional analysis of different alleles must be explored further.

There is functional data to suggest HLA-B*51^{+}MICA*009^{+} target cells inhibit cell mediated cytotoxicity from BD-derived PBMC in an unpredictable way (Figure 1-6), although the mechanism for this remains unclear. Of note, HLA-B52^{+}MICA*009^{+} cells do not behave in a similar manner when exposed to the same PBMC. Work from Shafi et al. (Shafi et al., 2011) demonstrated that polymorphisms within *MICA* do not cause varied levels of killing, but polymorphisms within *KIR3DL1* have yet to be closely examined in this context.

1.7.4 Functional Activity Of Natural Killer Cells In Behçet's Disease

In order to investigate the functional activity of NK cells in BD, Yamaguchi et al. assessed 47 patients with BD, (10 with active disease). CD69^{+} NK cells were significantly increased in active BD, but their cytotoxic function was similar to inactive and control subjects the investigators also reported that IL-12Rβ2 mRNA levels were decreased in NK cells from patients with active BD, compared to inactive patients or healthy controls.

Furthermore, NK cells from inactive patients suppressed IFNγ production by CD4^{+} T cells from patients with active disease (Yamaguchi et al., 2010). These findings suggest that while NK cells proliferate in active BD, they may be rendered hyporesponsive due to an abnormality in IL-12 signaling.

Hamzaoui et al. also described hyporesponsive NK cells in bronchoalveolar lavage samples from patients with pulmonary BD. The authors did not examine the effects of IL-12, but instead focused on IL-2/IL-15Rβ (CD122), which was found to have reduced surface expression. CD122 is shared by the IL-15 receptor and is partly responsible for maintenance and cytotoxicity of NK cells (Hamzaoui et al., 2013).

In 2017, Hasan et al. phenotyped the NK cell compartment in a cohort of 60 individuals with BD and 60 healthy controls. The investigators identified that circulating NK cells were significantly reduced in the BD group. In addition, active disease was correlated with reduced numbers of circulating NK cells. In a study examining the effects of Herpes Simplex Virus (HSV) in BD, Hamzaoui et al. noted that both NK cell activity and the number of NK cells was found to be reduced in BD, despite an elevation in IFNγ titres and the presence of activated T cells (Hamzaoui et al., 1990).

Conversely, in a study evaluating the effect of IFNα-2a on lymphocytes populations in BD, Treusch et al. reported increased percentages of $CD56^{+}CD16^{+}$ NK cells in PBMC from active BD patients compared to healthy controls prior to treatment. Interferon-α-2a is often used as a second or third line therapeutic agent in BD, particularly in the presence of ocular disease. The investigators found a significant decrease in circulating NK cells in active BD patients after treatment compared to controls. This reduction may be a direct effect of IFNα-2a or secondary to induction of remission of BD. IFNα-2a has been postulated to work directly on NK cells by inducing apoptosis and indirectly by reducing IL-18, IL-12 and/or IL-21 (Treusch et al., 2004).

Takeno et al. studied 40 Japanese patients with BD (60% had ocular disease) and noted that 10% had increased levels of circulating NK cells compared to normal controls. The authors characterised NK cells and found that the expression of KIR3DL1 in the BD group was highly variable (12.99±12.49%) compared to controls (16.5±6.4%). The authors also looked at KIR3DL1 and Bw4 expression, but found no differences between $Bw4^{+}$ and Bw4- patients, which is in keeping with previous findings (Gardiner et al., 2001; Gumperz et al., 1995). They hypothesised that defects in the NK cell repertoire may permit persistent viral infection leading to BD or that NK cells lacking appropriate

inhibitory KIR (such as KIR3DL1) may fail to recognise self-MHC and cause autologous tissue damage (Takeno et al., 2004).

1.8 Chapter Summary

Behçet's Disease is a multisystem disease, which mainly affects young people of working age. It can manifest in a variety of clinical phenotypes and is associated with significant morbidity and mortality. There is no cure for BD and current management is chiefly based around long-term immunosuppression.

The pathogenesis of BD remains unknown. Recent large-scale GWAS have identified a number of susceptibility loci, both in and out of the MHC. The candidate genes mentioned in Table 1-8 are likely to represent the 'tip of the iceberg' and more rare variants will be uncovered over the coming years each with a diminishing effect size (Ombrello, Sikora, et al., 2014). Despite being described more than 30 years ago, the majority of disease hereditability comes from *HLA-B*51*, the role of which remains unclear.

KIRs regulate cells of the innate immune system and are either activating or inhibitory. The KIR3DL1/S1 gene is highly polymorphic and 110 alleles have been described (https://www.ebi.ac.uk/ipd/kir/stats.html) to date. Furthermore, KIR3DL1 is known to interact with the Bw4 epitope of HLA-B*51, however, no work has been done looking at KIR3DL1 variants and their inhibitory relationship to HLA-B*51. Previous work from our group suggests that polymorphisms within KIR3DL1 may contribute to an alteration in cell mediated cytotoxicity, most likely via the interaction with Bw4. This alteration may lead to a positive feedback loop causing prolonged inflammation and a chronic inflammatory state; this is the focus of my thesis (Figure 1-9).

While such interactions alone will not elucidate the pathogenesis of BD, it is hoped that they will clarify key aspects of human NK cell regulation that will be of benefit to understanding many aspects of human cell-mediated immune responses in health as well as in disease.

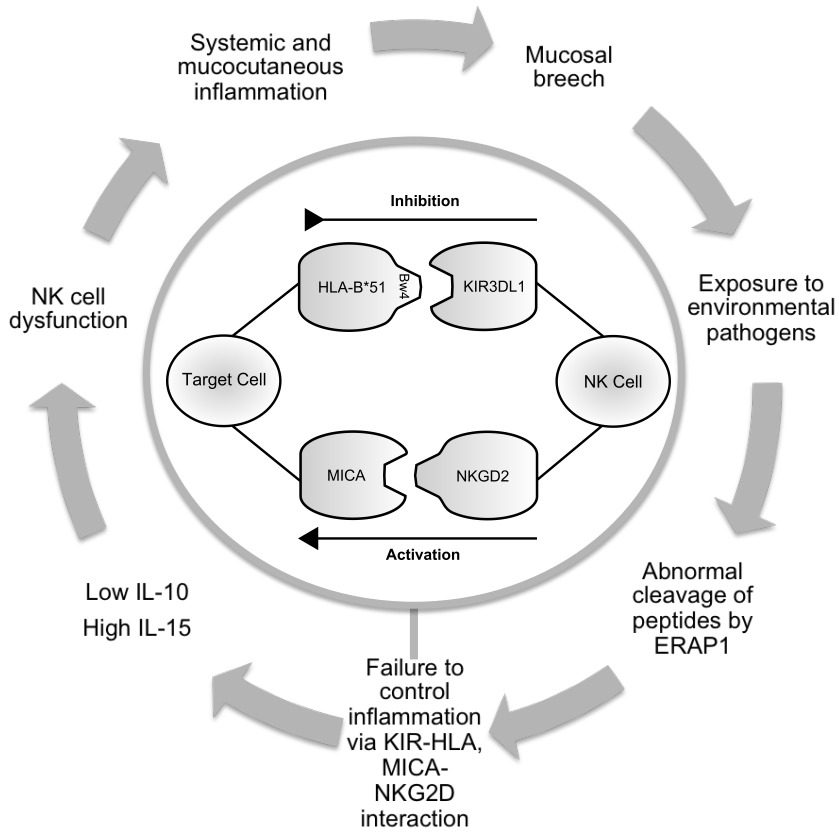
1.9 Hypothesis

The working hypothesis of this thesis is that specific *KIR3DL1/S1* alleles are associated with BD. If this is the case, then I further hypothesise that certain KIR3DL1/S1 allotypes show varied levels of inhibition to cell-mediated killing when presented with their ligand, HLA-B*51.

Experimental support for this hypothesis would add weight to the prospect that disease-associated alleles of *KIR3DL1/S1* may contribute to lymphocyte dysregulation in BD. Conversely, refuting the hypothesis may reduce support for this perspective.

Figure 1-9 Putative Role For KIR3DL1-HLA-B*51 Interaction Causing A Prolonged Chronic Inflammatory State In Behçet's Disease

*Infection or inflammation at mucocutatneous surfaces may persist due to dysfunction of the innate immune response in predisposed individuals. ERAP1 may have a role in processing peptides that are presented by HLA-class-I molecules. KIR3DL1 inhibits cell-mediated cytotoxicity via interaction with HLA-B*51. This effect is balanced by activating interactions via NKG2D and MICA. Production of cytokines including IL-10 and IL-15 contribute to abnormal NK cell function leading to prolonged inflammation and further episodes. NK cell, Natural killer cell; ERAP, endoplasmic reticulum aminopeptidase 1; HLA, human leukocyte antigen; KIR, killer immunoglobulin-like receptor; MICA, MHC class-I polypeptide-related sequence A; NKG2D, Natural killer group 2; receptor D; IL, interleukin. Reproduced with permission from (Petrushkin et al., 2015)*



1.10 Aims Of The Thesis

1. To investigate whether established HLA and MICA associations are applicable to a large cohort of individuals with BD from the UK.

2. To establish whether specific *KIR3DL1/S1* alleles are associated with BD.

3. To investigate the expression of KIR3DL1/S1 in a group of individuals with BD with known KIR3DL1/S1 alleles.

4. To create a group of cell lines expressing relevant HLA-B ligands to determine the functional effects of KIR3DL1/S1 in vitro.

5. To determine whether HLA-B*51-KIR3DL1/S1 interaction results in increased degranulation compared to HLA-B*52-KIR3DL1/S1 interactions.

6. To investigate the incidence and phenotypic heterogeneity of ophthalmic BD in the UK.

2 Materials and Methods

2.1 Ethics, Consent And Study Participants

This project was carried with institutional ethics approval (Immunoregulation at the mucosal barrier, P/03/122) granted by the east London & the city research ethics committee. Individuals fulfilling the ISG criteria for BD (Disease, 1990) and unrelated, demographically matched controls, were approached in the London BD Centre of Excellence. Patients and controls were given information regarding the study and a consent form to read and take away with them. On their next visit, patients and healthy controls were consented and given a research number, which was used to identify that individual for the rest of the study. Original consent forms were held in a specified, locked cabinet in a locked room in a secure building (Blizard Institute, Queen Mary University of London (QMUL)). Patients with BD were recruited regardless of their age, whereas HCs were recruited over the age of 45 wherever possible to avoid the possibility of incorrectly labelling an individual as 'healthy' if they had an unmasked auto-inflammatory disease (discussed further in Chapter 3.3).

2.2 Power

Calculating the power required in this project was challenging due to the rare nature of the disease in the UK as well as the polymorphic nature of HLA and KIR. In particular, *KIR3DL1* alleles have not been explored in a BD population.

I used the population prevalence of *KIR3DL1*001* in the UK as a basis for the power calculation. *KIR3DL1*001* is found in approximately 15% of the UK population (Norman et al., 2007b). I assumed a prevalence of 7.5% in the control group and planned a

recruitment ratio of 1:2 BD:HC. In order to achieve a power of 80% with an α of 0.05, 234 individuals with BD and 468 HCs needed to be recruited. Power calculations were carried out using clincalc.com power calculation tool for two independent samples with a dichotomous endpoint (www.clincalc.com/stat). As *KIR3DL1* is not a dichotomous allele, it is unlikely that this power calculation will be accurate, and it would be ideal to collect a larger number of cases and controls to allow for contingency.

2.3 Venepuncture And Blood Product Handling

Blood was taken from participants in a clean-room in the BD clinic using appropriate equipment and safety precautions. Consent was re-affirmed prior to venepuncture. For DNA extraction, 10 millilitres (ml) of blood was taken supplemented with the anticoagulant; ethylenediaminetetracetic acid (EDTA). For PBMC analysis, 40 ml of blood was taken.

The blood bottles were labelled with the participants' research number and taken to the Blizard Institute for DNA/PBMC extraction. DNA was extracted within 1 week of venepuncture. During that time, the blood bottles were stored at 4 degrees Celsius (°C). PBMC were extracted immediately.

2.4 Molecular Techniques

2.4.1 DNA Extraction

DNA was extracted using a QIAamp DNA Blood Maxi-Kit (Cat No. 51192, Qiagen, Hilden, Germany). Manufacturer's instructions were followed. 10 ml of anti-coagulated

blood was brought to room temperature and vortexed before being mixed with 500ul Qiagen protease in a 50 ml labelled, skirted conical tube. Twelve millilitres of Buffer AL (lysis buffer) was then added and shaken for 1 minute (min). The tube was then incubated at 70°C for 10 minutes (min) in a water bath. 10 ml of 100% ethanol was then added to the contents of the 50 ml conical tube and shaken vigorously for 1 min.

A QIAgen maxi-column was then prepared and labelled. Half the contents of the 50ml conical flask was transferred to the membrane and centrifuged at 1,850 times gravity (g) for 3 mins. The eluent was then discarded and the remainder added to the membrane and centrifuged at 1,850g for a further 3 min.

To wash the membrane, 5 ml of Buffer AW1 was applied to the column and left to incubate at room temperature for 5 mins prior to centrifuging at 4,500g for 1 min. This was followed by 5 ml of Buffer AW2 being applied to the membrane and immediately centrifuged at 4,500g for 15 mins, to ensure all ethanol was removed from the membrane.

To elute the sample, a freshly labelled 50 ml conical tube was prepared and the maxi-column inserted. 1 ml of Buffer AE was added to the membrane and incubated at room temperature for 5 mins. The sample was then centrifuged at 4,500g for 2 mins. An addition 1ml of Buffer AE was then added to the membrane and incubated for 5 mins at room temperature before being centrifuged at 4,500g for 2 mins. The resulting 2 ml of eluent was then vortexed and measured for DNA concentration. A Nanodrop nanospectrometer (ThermoFisher, Waltham, MA, USA) was used to quantify DNA concentration. A 260/280 ratio of 1.8 was considered the target for DNA purity. DNA was stored at 100 nanograms per microliter (ng/ul) and either diluted with Buffer AE or concentrated using a heated centrifuge with a vacuum applied. DNA was labelled both with a permanent

marker and a label designed to withstand -80°C temperature. DNA was then stored at -80°C.

2.4.2 Library Preparation And Bioinformatics Pipeline

Primers were designed for HLA-B, MICA, KIR3DL1 and a panel of 25 ancestry informative markers (AIMs) by Charles Mein at the Genome Centre in QMUL. The DNA samples were transported to the Genome Centre and the library preparation was carried out by Thedoros Xenakis on a Fludigm Platform (Fluidigm, South San Francisco, CA, USA). Sequencing was carried using a MiSeq platform (Illumina, San Diego, CA, USA) at the Genome Centre. *HLA-B* alleles were assigned using NGSengine software (GenDX, Utrecht, the Netherlands) and *KIR3DL1/S1* alleles were assigned using the PING pipeline (Norman et al., 2016).

2.4.3 mRNA Extraction

mRNA was extracted using the Dynabeads mRNA DIRECT purification kit (Cat No. 61012, ThermoFisher, Waltham, MA, USA) according to manufacturer's instructions. $1x10^6$ cells (either from cell culture or PBMC) were pelleted at 400g for 8 mins. The pellet was washed with phosphate buffered saline (PBS) and centrifuged again at 400g for 8 mins. 300ul of lysis/binding buffer was then added and gently pipetted until the pellet had dissolved on ice. The lysate was then forced through a 21-gauge needle 3 times using a 2 ml syringe to shear DNA and reduce viscosity.

Dynabeads were vortexed prior to use and 50ul of beads were placed in a sterile tube on a DynaMag-2 magnet (Cat No. 12321D, ThermoFisher, Waltham, MA, USA). The

supernatant was then removed and the beads washed with 100ul of Lysis/Binding Buffer. The beads were then put back on the magnet and the supernatant aspirated. 300ul of lysate was then added to the beads and the sample gently pipetted to mix the contents. The lysate and beads were then put on a tube roller and gently rotated for 10 mins at room temperature to allow hybridization of the beads.

The tube containing the hybridized beads was then placed back on the magnet for 2 mins and the supernatant removed. The beads were then washed twice with 400ul Washing Buffer A at room temperature, using the magnet to separate the beads between each wash. The beads were then washed once with 400ul of Washing Buffer B at room temperature and the magnet used to separate the supernatant for aspiration.

To remove mRNA from the beads, 13ul of Elution Buffer was added to the beads and incubated at 80°C for 2 mins. The beads were then placed back on the magnet and the supernatant removed to a fresh labelled PCR 0.2ml tube on ice.

2.4.4 cDNA Preparation From mRNA

The Roche Transcriptor First cDNA Synthesis Kit (Cat No. 04379012001, Roche, Basel, Switzerland) was used according to manufacturer's instructions. A reverse transcription (RT) master mix of 4.0ul 5x RT Buffer, 2.0ul deoxyribose nucleoside triphosphates (dNTP) mix (10uM), 0.8ul Random OligoDT mix, 0.4ul RNAse inhibitor and 0.4ul Reverse Transcriptase was made during the lysis incubation period of mRNA extraction. 7ul of RT master mix was added to 13ul of mRNA in a 0.2ml PCR tube. The following RT settings were used on a Veriti (ThermoFisher, Waltham, MA, USA) thermocycler (Table 2-1).

Table 2-1. Thermocycler Settings For cDNA Synthesis.

Temp	Time (mins)	Repeat
42°C	30.00	1x
85°C	5.00	1x
4°C	5.00	1x

The cDNA was then diluted with 50ul nuclease free (NF) water and stored at -20°C for use in downstream applications.

2.5 HLA Analysis

HLA-B analysis was carried out using the NGS Engine Software package (GenDx, Utrecht, Netherlands). Samples were analysed in collaboration with the Parham Laboratory, Department of Structural Biology, Stanford University, CA, USA. HLA-A was analysed using sequence specific oligonucleotide probes (SSOP) (Immucor, Peachtree Corners, GA, USA). There were 28 HLA-B samples with ambiguous or unlikely reads which were also confirmed with SSOP.

For the amplification, DNA was normalised to 50ng/ul and 2ul were added to a master mix comprised of: 13.5ul nuclease free (NF) water (Cat No. W4502-1L, Sigma Aldrich, St Louis, MI, USA), 6.6ul Loci specific master mix (HLA-A or B), 0.2ul Taq polymerase (Cat No. M7806, Promega, Madison, WI, USA). Thermocycler settings can be seen in (Table 2-2). After amplification, the 2ul of amplified DNA was added to 8ul of Loci-Specific Probes and the sample was hybridised on a thermocycler with the following settings (Table 2-3).

Table 2-2. Thermocycler Settings For *HLA-A* And *B* Amplification: Sequence Specific Oligonucleotide Probe (SSOP) Protocol.

Temp	**Time**	**Cycles**
95.0°C	3:00	1x
95.0°C	0:15	12x
60.0°C	0:30	
72.0°C	0:30	
95.0°C	0:10	28x
63.0°C	0:30	
72.0°C	0:30	
72.0°C	2.00	5x
20.0°C	∞	1x

Table 2-3. Thermocycler Settings For Hybridisation Of SSOP Probes To DNA

Temp	**Time**	**Cycles**
97.0°C	2:00	1x
47.0°C	10:00	1x
56.0°C	8.00	1x
56.0°C	∞	1x

After 8 minutes at 56°C, but while the sample is still on the thermocycler, the adhesive lid was removed from the plate and 20ul of SAPE buffer was added. The plate was then moved to a Luminex Liquichip 200 (Luminex, Austin, TX, USA) for analysis. Data was then exported to MatchIT DNA (Immucor, Peachtree Corners, GA, USA) for typing.

2.6 Agarose Gel Electrophoresis

Visualisation of amplified DNA products from PCR reactions was performed by agarose gel electrophoresis in the presence of 0.001% SYBR-Safe (Cat No. S33102, ThermoFisher, Waltham, MA, USA). To make the gel, the appropriate amount of agarose

was weighed and added into an appropriate volume of 1X Tris-Acetate EDTA (TAE) buffer (Cat No. B49, ThermoFisher, Waltham, MA, USA) to make a 1% TAE agarose gel and then microwaved for 2-3 minutes to fully dissolve the agarose in the 1X TAE buffer. This was followed by the addition of 0.001% SYBR-Safe before being poured in to a gel mould. The gel was then allowed to solidify before being placed in a gel tank containing sufficient TAE buffer to completely cover the gel. Samples were then combined with 6x Gel Loading Dye Blue (Cat No. B7021S, New England Biosystems (NEB), Ipswich, MA, USA), loaded into the gel and run through at approximately 90-300V for 30-60 minutes, before visualization of DNA bands under ultraviolet light. Different size gels were used for different applications. Generally, a small 50ml gel was used for DNA analysis and cloning, whereas a large 1400ml gel was used for HLA-typing.

2.7 Polymerase Chain Reaction Single Specific Primer (PCR SSP)

PCR SSP was used for HLA-typing if there was an ambiguous result from SSOP. Primers were based on those described by Bunce et al (Bunce et al., 1995).

2.7.1 *HLA-A* Typing SSP

22.5ul of DNA at 50ng/ul was added to the following master mix: 130ul of NF water (Cat No. W4502-1L, Sigma Aldrich, St Louis, MI, USA) 52.5ul Buffer (Cat No. M7806, Promega, Madison, WI, USA), 17.5ul 63/64 control DNA product (Eurogentec, Liege, Belgium):

Forward Primer 5'-3' (FP) - TGCCAAGTGGAGCACCCAA

Reverse Primer 5'-3' (RP) - GCATCTTGCTCTGTGCAGAT

26.25ul dNTPs (Cat No. R0182, ThermoFisher, Waltham, MA, USA), 12.25ul Magnesium Chloride ($MgCl_2$) (Cat No. M7806, Promega, Madison, WI, USA), 2ul Taq polymerase (Cat No. M7806, Promega, Madison, WI, USA). The same was then vortexed and aliquoted into 25 wells with HLA-A primers pre-seeded and dried. The plate was then sealed and placed in a thermocycler (Table 2-4).

Table 2-4. Thermocycler Settings For *HLA-A* And *HLA-B* PCR SSP Analysis.

Temp	**Time (min)**	**Cycles**
94.0°C	1:00	1x
94.0°C	0:25	5x
70.0°C	0:45	
72.0°C	0:30	
94.0°C	0:25	20x
63.0°C	0:45	
72.0°C	0:30	
94.0°C	0:25	5x
55.0°C	1:00	
72.0°C	2:00	
20.0°C	∞	

The samples were then loaded into a 1% agarose gel and run as detailed above. The DNA image was then captured under ultraviolet (UV) light and analysed.

2.7.2 *HLA-B* Typing SSP

47ul of DNA at 50ng/ul was added to the following master mix: 260ul of NF water, 109ul Buffer, 55ul dNTPs, 47ul 63/64 control DNA, 26ul $MgCl_2$, 4.5ul Taq polymerase. The same was then vortexed and aliquoted into 50 wells with HLA-A primers pre-seeded and dried. Thermocycler settings were the same for HLA-A and B and can be found in (Table 2-4). Primers for HLA-Typing can be found in Appendix 1.

2.8 Construction of Cell Lines

Two cell lines were used in this project. The Chinese Hamster Ovary (CHO)-Flp-In cell line (Cat-No. R75807, ThermoFisher, Waltham, MA, USA), was obtained to use as a control cell line for cloning analysis and the 721.221 lymphoblastoid cell line, which is typically used in NK-cell cytotoxicity assays. The benefits and challenges of each cell line are listed in Table 2-5.

Table 2-5. Benefits And Challenges Of Chinese Hamster Ovary (CHO) Epithelial Cell Line And 721.221 Lymphoblastoid Cell Line

CHO – Chinese Hamster Ovary, HLA – Human Leukocyte Antigen, KIR – Killer Immunoglobulin-like Receptor, NK – Natural Killer, B2M – Beta 2 Microglobulin

	Benefits	Challenges
CHO-cell line	• Easy to transfect with the Flp-recombinase system (see Chapter 2.10) • Easy to grow • No innate expression of ligands for HLA or KIR – so effects of HLA transfection can be studied in isolation	• Cytotoxicity assays are challenging as the cell line is adherent • Not routinely used as a target cell line for NK cytotoxicity
721.221 cell line	• Easy to grow • Routinely used for NK cytotoxicity assays • Expresses HLA constructs due to innate expression of B2M	• Harder to transfect • Harder to sort cells using antibiotic resistance due to their non-adherent nature • Harder to obtain stable expression using transfection, rather than transduction techniques.

The CHO-MICA*009 cell line was obtained from the Hayday Laboratory, Immunobiology, Guy's Hospital, London, UK with thanks. The 721.221 (221) cell line

was obtained from the Moffat Laboratory, Centre for Trophoblast Research, Cambridge University, Cambridge, UK with thanks.

Eight cell lines were constructed in this project, not all of which were used in the final analysis.

1. pcDNA5- MICA*009/B2M/HLA-**B*51**- hygromycin resistant
2. pcDNA5- MICA*009/B2M/HLA-**B*52**- hygromycin resistant
3. pcDNA5- MICA*009/B2M/HLA-**B*35**- hygromycin resistant
4. pcDNA5-MICA*009 – hygromycin resistant
5. pcDNA3.1-HLA-**B*51** – neomycin resistant
6. pcDNA3.1-HLA-**B*52** – neomycin resistant
7. pcDNA3.1-HLA-**B*35** – neomycin resistant
8. pcDNA3.1-B2M – Zeocin resistant

The construction of each cell line is detailed below. The challenges encountered whilst constructing the cell lines is detailed in Chapter 4.

2.8.1 pcDNA5-FRT/MICA*009/B2M/HLA-B CHO-Cell Line

Throughout this chapter, the same primers and techniques were used for *HLA-B*51/*52/*35.* Thus, I have referred generically to 'HLA-B' when referring to these amplicons.

A pcDNA5-FRT vector (Cat-No. R75807, ThermoFisher, Waltham, MA, USA) encoding hygromycin resistance was used to transfect the triple construct into the CHO-Flp-In cell

line. MICA*009 was amplified from a pre-existing CHO-MICA*009 cell line using primers designed to amplify the whole coding DNA sequence (CDS):

MICA*009 FP – TTCCTGCTTCTGGCTGGCAT
MICA*009 RP – TAGGCGCCCTCAGTGGAG

The coding region of B2M was amplified from a healthy control individual with specific primers:

B2M FP – TCTCGCTCCGTGGCCTTAGC
B2M RP – AGCAATTCAGGAAATTTG

The *HLA-B*51:01:01* (henceforth *HLA-B*51*), *B*52:01:01* (henceforth *HLA-B*52*) and *B*35:01:01* (henceforth *HLA-B*35*) used in each construct were synthesised to ensure accuracy (Genscript, Nanjing, China). HLA-B was amplified using the following primers:

HLA- B FP – ACAACCATGCGGGTCACG
HLA- B RP – AGAGCCCTGGGCACTGTC

*2.8.1.1 Amplification Of Template MICA*009, B2M And HLA-B cDNA*

1ul of HLA-B, MICA*009, B2M and pcDNA5-FRT vector CDNA were separately normalised to 50ng/ul was added to 1.25ul F primer, 1.25ul R primer, 12.5ul Q5 high fidelity polymerase (Cat No. M091S, NEB, Ipswich, MA, USA), 9ul NF water in a PCR tube (Table 2-6).

Table 2-6. Thermocycler Settings For Amplifying Fragments For The Triple Construct

Melting and annealing temperatures were adjusted for different size products

Temp	**Time**	**Cycles**
98°C	0:30	1x
98°C	0:05-0:10	30x
64-69°C	0:20-0:25	
72°C	0:10	
72°C	5:00	1x

2.8.1.2 Purification Of Amplified cDNA Fragments

The amplified products were then run on a 1% agarose gel with a 2-log ladder (Cat No. N0550S, NEB, Ipswich, MA, USA) and visualised under UV light. The relevant bands were then cut out of the gel and weighed.

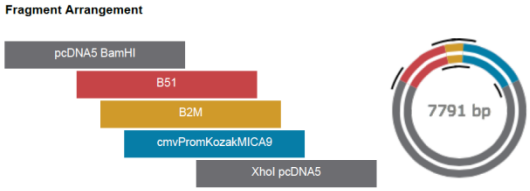
After weighing, DNA was extracted using the QIAquick Gel Extraction Kit (Cat No. 28704, Qiagen, Hilden, Germany). Three times volume of Buffer QG was added (100mg ~ 100ul, i.e. 300ul Buffer QG) and the mixture incubated at 50°C for 10 mins until the agarose had completely dissolved. The sample was vortexed and 1 gel volume of isopropanol (Cat No. I9516, Sigma Aldrich, St Louis, MI, USA) was added followed by a further vortex. The sample was then applied to a mini spin column and spun at 400g for 1 min. The eluent was then reapplied to the column two further times to ensure maximum binding of the DNA product to the silica membrane. To wash the membrane, 750ul of Buffer PE was added to the column and left to incubate at room temperature for 1 minute. The sample was then vortexed at 13,000g for 1 min. Finally, the spin-column was placed in a clean, labelled collection tube and 50ul of NF water was added and left to incubate for 1 minute at room temperature before centrifuging at 13,000g for 1 min. The DNA product was then measured using a spectrophotometer and kept at -20°C until use.

2.8.1.3 Gibson Assembly

*MICA*009, B2M* and *HLA-B* (henceforth referred to as the triple construct), was assembled using Gibson assembly. Gibson primers were designed using the Gibson Assembly designer (NEB, Ipswich, MA, USA). The stop-codon on the B2M CDS was removed and replaced with an internal ribosomal entry site (IRES) sequence built onto the end of the B2M CDS. Both the B2M and HLA-B CDS were then driven from an upstream cytomegalovirus promoter (pCMV) sequence. An additional pCMV was added immediately after the HLA-B sequence and before the MICA*009 sequence to ensure adequate expression of MICA*009 (Figure 2-1).

Figure 2-1. Gibson Assembly Protocol and Primer Design for CHO *MICA*009/B2M/ HLA-B* Cell Line

*http://nebuilder.neb.com allows users to upload fragment sequences and calculates the primers and annealing temperatures required to carry out Gibson Assembly. Below is a screen shot for the triple construct with HLA-B*51. Details of the construct can be seen in Figure 4-7.*



Required Primers

Overlaps	Oligo (Uppercase = gene-specific primer)	Anneals	F/R	3' Tm	3' Ta *	6-Frame
pcDNA5	ttggtaccgagctcgATGCGGGTCACGGCGCCC	B51	Fwd	75.8°C	**72.0°C**	view
B2M	cgagacatTCAAGCTGTGAGAGACACATCAGAGCCC	B51	Rev	72.2°C	**72.0°C**	view
B51	cagcttgaATGTCTCGCTCCGTGGCC	B2M	Fwd	68.6°C	**66.7°C**	view
cmvPromKozakMICA9	ccatttgcTTACATGTCTCGATCCCACTTAACTATC	B2M	Rev	63.7°C	**66.7°C**	view
B2M	gacatgtaaGCAAATGGGCGGTAGGCG	cmvPromKozakMICA9	Fwd	68.1°C	**70.5°C**	view
pcDNA5	acgggccctctagacCTAGGCGCCCTCAGTGGA	cmvPromKozakMICA9	Rev	67.5°C	**70.5°C**	view

Gibson assembly was carried out according to the manufacturer's instructions (Cat No. E5510, NEB, Ipswich, MA, USA). The four fragment (vector, B2M/IRES, HLA-B, pCMV/MICA*009) assembly was carried out at a ratio of 1:1:1:1. Each fragment was normalised to 0.2pmol and mixed in a 1.5ml Eppendorf before drying down in a vacuum

concentrator (DNA Speedvac, Cat No. DNA120-115, ThermoFisher, Waltham, MA, USA). The sample was then resuspended in 5ul NF water and 2ul Gibson Assembly Mix (Cat No. E26112, NEB, Ipswich, MA, USA). The sample was then incubated at 50C for 1 hour.

2.8.1.4 Transformation Into E. Coli

pcDNA5-MICA*009/B2M/HLA-B was transformed into NEB 5 α competent *E. Coli* (Cat No. C2987I, NEB, Ipswich, MA, USA). 2ul of vector was added to 25ul of NEB 5 α in an Eppendorf tube on wet ice and incubated for 30mins. A heat shock was carried out at 42C for 30 seconds followed by another 2-minute incubation on wet ice. 1ml of super optimal broth with catabolite repression (SOC) (Cat No. S1797, Sigma Aldrich, St Louis, MI, USA), media was then added to the *E. Coli* and the sample incubated for 1 hour at 37°C.

2.8.1.5 Plating And Selection Of Colonies

The transformed, chemically competent *E. Coli* were then plated on to agar plates supplemented with 0.2% carbenicillin (Cat No. C1389- Sigma Aldrich, St Louis, MI, USA). The sample was gently pipetted to mix the bacteria and then 100ul was plated onto a 10cm labelled, agar plate and evenly spread using sterile glass beads (Cat No. Z143928, Sigma Aldrich, St Louis, MI, USA). The remainder of the sample was then centrifuged at 13,000g for 1 minute, the supernatant removed and the pellet resuspended in 100ul of SOC. This was then added to a second labelled plate. The same procedure was carried out with the digested vector to serve as a control. The plates were incubated in the dark at 37°C for 12-16 hours before colonies were picked.

The following day, four colonies were punched out of the agar using a 200ul pipette tip. The tip was then submerged in 10 ml of Luria-Bertani (LB) broth (Cat No. L3022, Sigma Aldrich, St Louis, MI, USA) supplemented with 0.2% carbenicillin. The sample was then incubated on a shaker at 37°C for 16hrs.

2.8.1.6 Isolation Of Plasmid DNA (Miniprep)

The bacterial suspension was removed from the shaker and centrifuged at 13,000g for 10mins at room temperature. Plasmid DNA was isolated using the QIAprep miniprep kit (Qiagen, Hilden, Germany). The supernatant and 200ul tip were discarded and the pellet resuspended in 1ml of P1 Resuspension Buffer + 100ug/ml RNase A. The suspension was transferred to a 1.5ml Eppendorf and centrifuged at 6,800g for 3 mins. The supernatant was removed and the pellet resuspended in 250ul P1 Resuspension Buffer +100ug/ml RNase A. 250ul of P2 Lysis Buffer was then added to the suspension and the sample rolled gently until fully lysed. The sample was then neutralised with 350ul N3 Neutralisation Buffer and rolled until two clear phases appeared in the tube. The sample was then centrifuged at 17,000g for 10 mins and the supernatant removed to a QIAprep spin column. The column was centrifuged at 17,000g for 1 minute and the eluent discarded. The membrane was washed with 500ul Buffer PB, centrifuged at 17,000g for 1 minute and the eluent discarded. 750ul of Buffer PE was then added to the column and centrifuged at 17,000g for 1 minute. The spin column was then centrifuged 'dry' at 17,000g for 1 minute to ensure all ethanol had been removed from the membrane. To elute the DNA, the spin column was transferred to a clean, labelled 1.5ml Eppendorf tube and 50ul of NF water was added and allowed to incubate at room temperature for 5 minutes. The sample was then centrifuged at 17,000g for 1 minute and the eluent measured on a spectrophotometer to establish concentration of plasmid DNA.

2.8.1.7 Sanger Sequencing Confirmation Of Inserts

Two companies were used to send sampled for confirmation (SourceBio, Nottingham, UK and GATC, Konstaz, Germany). All four samples were sent for sequencing. 500ng of plasmid DNA was diluted with 5ul water either a forward or a reverse primer to amplify the region of interest. Sequences were usually returned within 1-3 days and were analysed with SnapGene software (GSL Biotech, Chicago, IL, USA). Samples with any mutations were discarded.

2.8.2 pcDNA5-MICA*009

The triple construct did not express HLA-B (discussed further in Chapter 4), so I created separate vectors each encoding a different antibiotic resistance gene to allow drug selection for each construct. The pcDNA5-MICA*009 plasmid was already constructed and obtained from the Hayday laboratory and had a hygromycin resistance gene.

2.8.3 pcDNA3.1(+)-HLA-B

A pcDNA3.1(+) vector encoding neomycin resistance was used to clone HLA-B*51/*51/*35 (Figure 2-2). HLA-B fragments were amplified using the quantities described in Chapter 2.8.1.1 with the following primers. Thermocycler settings for amplification are in Table 2-7. The products were digested with HindIII HF and Not1 HF prior to ligation into the vector.

HLA-B FP – GCACTGAAGCTTATAGCCACCATGCGGGTCACGGCG

HLA-B RP – AGAGCCCTGGGCACTGTC

Table 2-7. Thermocycler Settings For Amplification Of HLA-B Fragment Prior To Restriction Enzyme Digestion

Temp	**Time**	**Cycles**
98°C	0:30	1x
98°C	0:05	30x
69°C	0:20	
72°C	0:10	
72°C	5:00	1x

2.8.3.1 Digestion Of HLA-B Amplicon

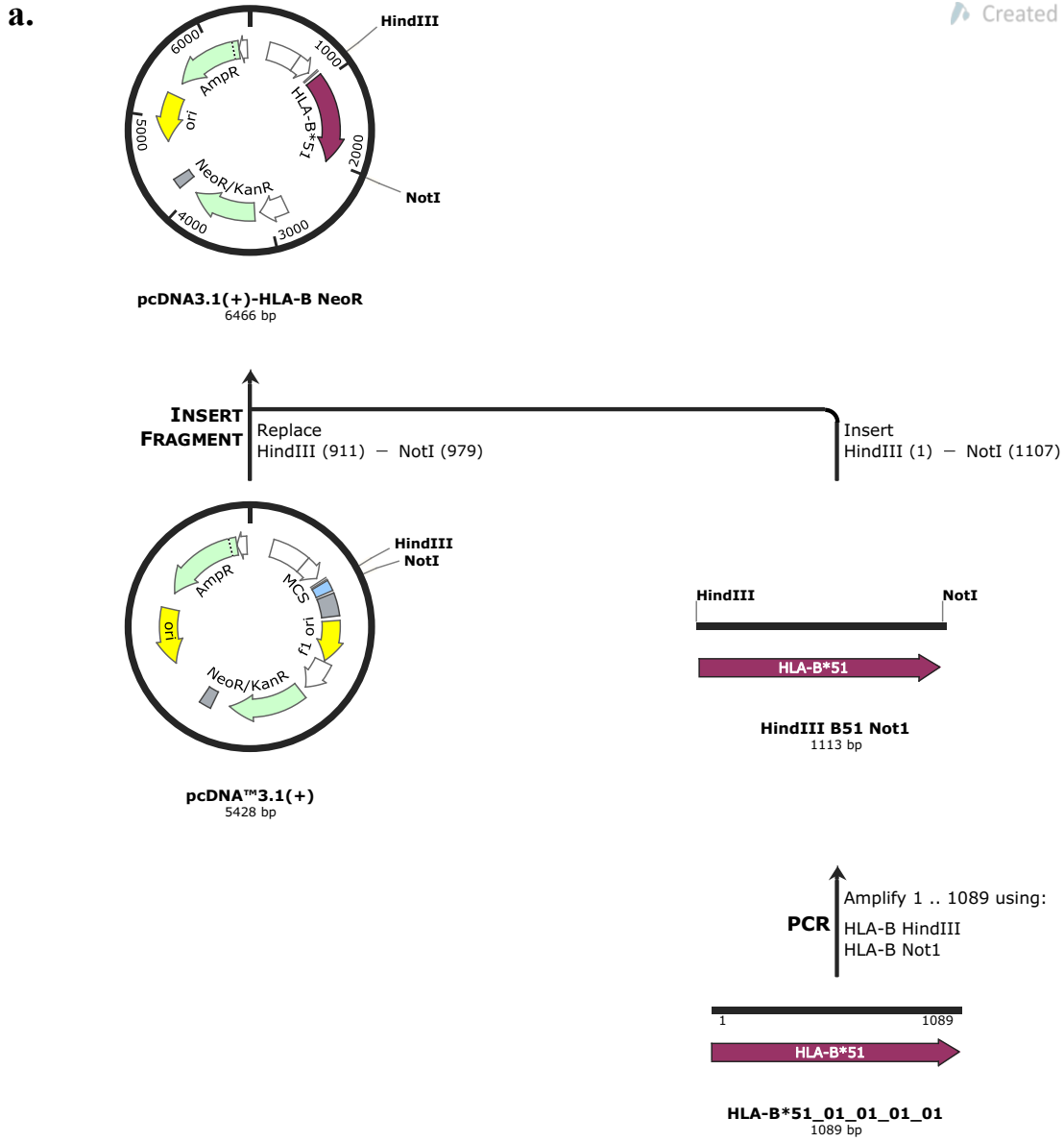
10ul of 50ng/ul (500ng) amplified product and vector were digested for 1 hour with 0.5ul HindIII HF, 0.5ul Not1 HF restriction enzymes (Cat No. R3104S and R3189L respectively, NEB, Ipswich, MA, USA), 2ul Cutsmart Buffer (Cat No. B7204S, NEB, Ipswich, MA, USA) and 7ul of NF water at 37°C.

In addition to the above, the digested vector was dephosphoralated using shrimp alkaline phosphatase (rSAP) (Cat No. M0371S, NEB, Ipswich, MA, USA) to prevent re-ligation of the cut ends. 1ul of rSAP was added to the digestion mix after 1 hour and left to incubate for a further 30 mins before being heat inactivated at 65°C for 5 mins.

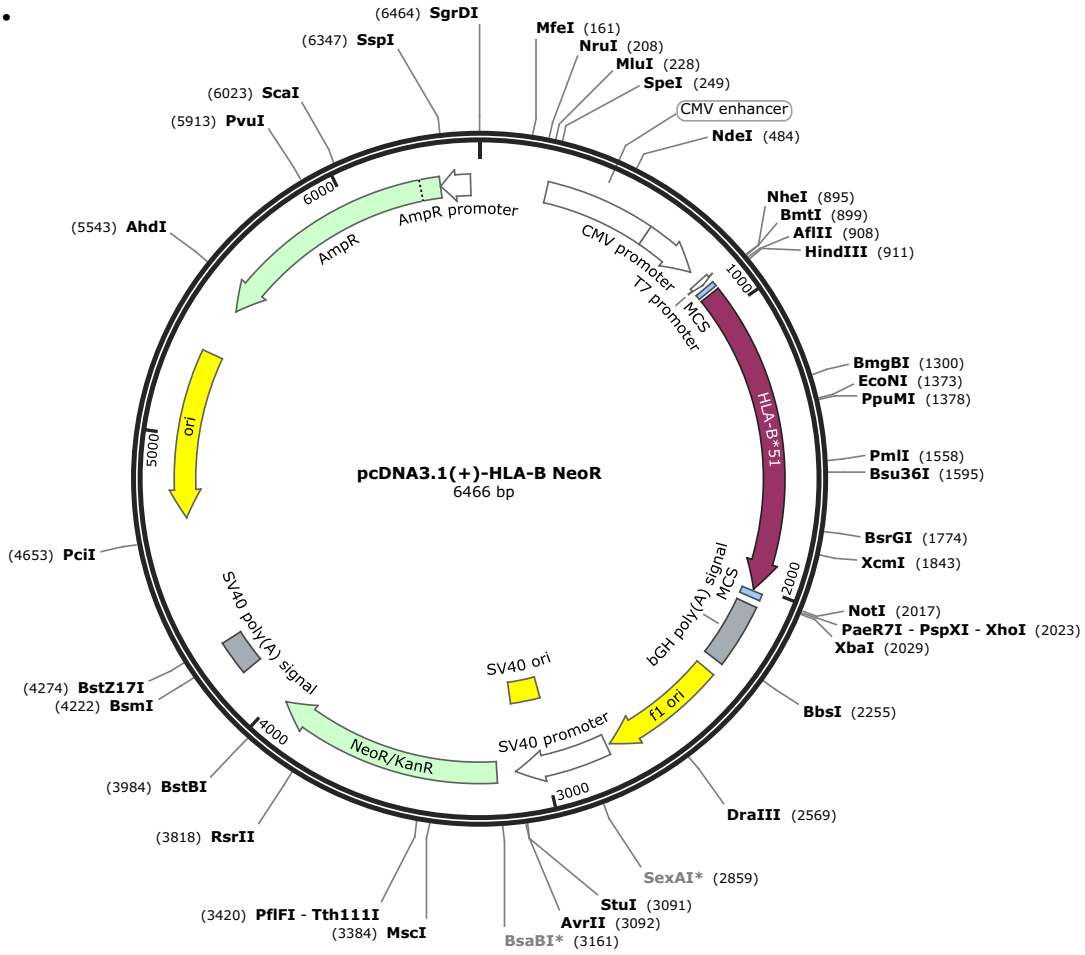
Figure 2-2. (a.) Method Used To Construct pcDNA(+) –HLA-B*51 With Neomycin Resistance Gene, (b.) pcDNA3.1(+) -HLA-B*51 In Detail

*(a.) Snapgene history to show amplification of synthesised HLA-B fragment with primers encoding HindIII and Not1 restriction sites. This was followed by insertion of the fragment by cutting and ligating the pcDN3.1(+) vector at the same restriction sites. (b.) Detail of the pcDNA3.1(+)-HLA-B*51 vector. Expression of gene of interest is driven by a CMV promoter immediately before the MCS. Expression of the neomycin resistance gene is driven by the SV40 promotor. HLA – Human Leukocyte Antigen, NeoR – neomycin resistance gene, AmpR – ampicillin resistance gene, PCR – polymerase chain reaction, CMV – cytomegalovirus, MCS – multiple cloning site.*

a.



b.



The digested vector and amplified HLA-B product was then run on an agarose gel, cut out and purified as described in Chapter 2.8.1.2.

2.8.3.2 Ligation

Ligation was carried out at a ratio of 3:1 (insert:vector). Ligations were carried out in combination with a control (vector only). 50ng/ul vector pcDNA3.1(+) in 4.2ul was added to 30ng insert HLA-B cDNA in 1.9ul, 2ul of T4 Ligase Buffer (Cat No. B0202S, NEB,

Ipswich, MA, USA), 1ul T4 Ligase (Cat No. B0202S, NEB, Ipswich, MA, USA) and 10.9ul NF water at room temperature for 30mins.

The sample was then transformed into *E. Coli*, plated out, colonies picked and plasmid DNA extracted as described in Chapters 2.8.1.4-2.8.1.7.

2.8.4 pcDNA3.1(+) - B2M

The zeocin resistance gene in the CHO-FRT cells was isolated by extracting mRNA from the CHO-FRT cells and synthesising cDNA as above. The Zeocin resistance gene was incorporated into the pcDNA3.1(+) vector using Gibson Assembly. B2M was then amplified and ligated into the pcDNA3.1(+) (Zeo) vector using restriction enzymes. Figure 2-3 outlines the methods used for creation of this plasmid.

2.8.4.1 Amplification Of Zeocin Resistance Gene From CHO-FRT cDNA And pcDNA3.1(+) Prior To Gibson Assembly

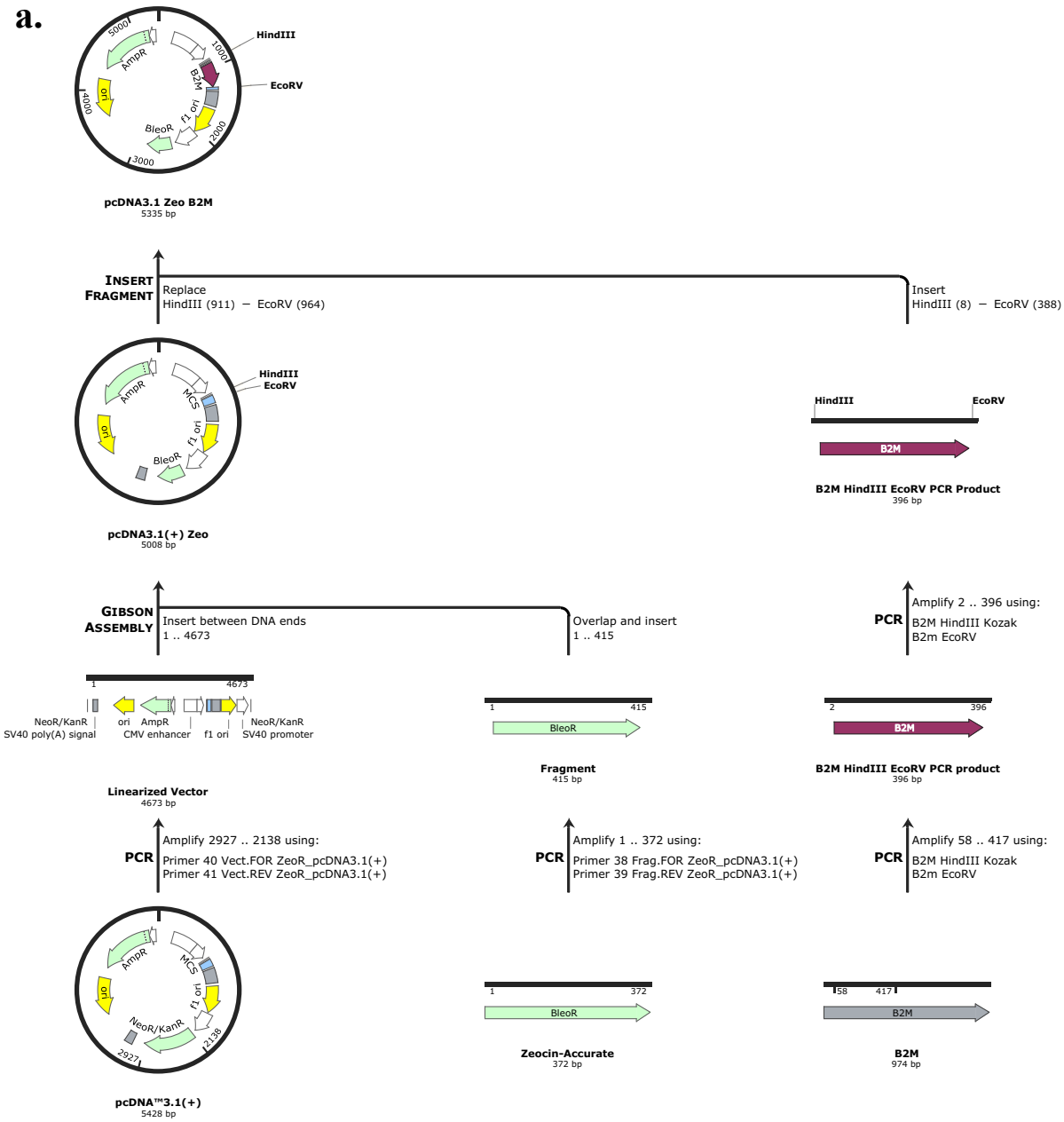
Specific primers to amplify zeocin were made and the fragment amplified using the settings in Table 2-8 and the quantities in Chapter 2.8.1.1

Zeocin FP – CAGGATGAGGATCGTTTCGCATGGCCAAGTTGACCAGTGC

Zeocin RP – TTCGAACCCCAGAGTCCCGCTCAGTCCTGCTCCTCGGC

Figure 2-3. (a.) Methods Used To Construct pcDNA3.1(+)–B2M with Zeocin Resistance Gene. (b.) Detail Of The pcDNA3.1(+)-B2M Vector

(a.) Snapgene history to show amplification of the Zeocin resistance gene and pcDNA3.1(+) vector with Gibson Assembly primers, followed by Gibson ligation of the two-fragments. The B2M gene was amplified with primers encoding the HindIII and EcoRV restriction sites and ligated into the plasmid. (b.) Detail of the pcDNA3.1(+)-B2M vector. Expression of gene of interest is driven by a CMV promoter immediately upstream of the MCS. Expression of the Zeocin resistance gene is driven by the SV40 promotor. HLA – Human Leukocyte Antigen, ZeoR/BleoR – Zeocin resistance gene, AmpR – ampicillin resistance gene, PCR – polymerase chain reaction, CMV – cytomegalovirus, MCS – multiple cloning site, B2M – Beta 2 microglobulin.



b.

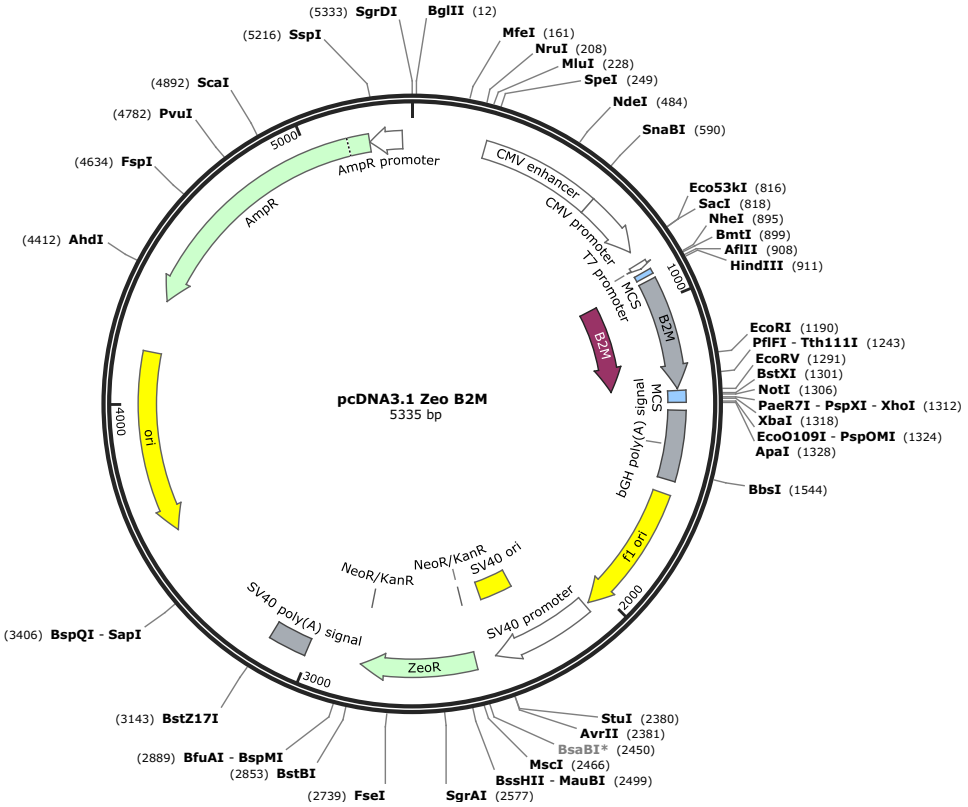


Table 2-8. Thermocycler Settings For Amplification Of Zeocin Resistance Fragment

Temp	Time	Cycles
98°C	0:30	1x
98°C	0:05	30x
70°C	0:20	
72°C	0:15	
72°C	5:00	1x

Specific primers to amplify the pcDNA3.1(+) vector were also designed and amplified using the settings in Table 2-9 and the quantities in Chapter 2.8.1.1.

pcDNA3.1(+) FP - TGGCCGAGGAGCAGGACTGAGCGGGACTCTGGG

pcDNA3.1(+) RP – GCACTGGTCAACTTGGCCATGCGAAACGATCCTCATCCT

Table 2-9. Thermocycler Settings For Amplification of pcDNA3.1(+) Prior to Gibson Assembly

Temp	**Time**	**Cycles**
98°C	0:30	1x
98°C	0:05	30x
69°C	0:20	
72°C	0:25	
72°C	5:00	1x

The Zeocin amplicon was then run on an agarose gel and purified over a silica membrane as detailed in Chapter 2.8.1.2. The pcDNA3.1(+) vector was also purified and then a 2 fragment Gibson assembly was carried out at a 3:1 (insert:vector) ratio. 50fmol vector mixed with 150fmol of insert and dried in a vacuum concentrator. The DNA was resuspended in 5ul of NF water, 5ul Gibson Assembly Mix and incubated at 50°C for 15 minutes. The sample was then transformed into *E. Coli*, plated out, colonies picked and plasmid DNA extracted as detailed in Chapter 2.8.1.4-2.8.1.7. The vector was sent for Sanger sequencing to check for mutations within the Zeocin resistance gene.

2.8.4.2 Amplification, Digestion And Ligation Of B2M Into The pcDNA3.1(+) Zeocin Resistant Vector

B2M was then amplified with primers encoding EcoRV and HindIII restriction sites using quantities described in Chapter 2.8.1.1 and thermocycler settings in Table 2-10.

B2M FP - GGGAAAAAGCTTATAGCCACCATGTCTCGCTCCGTG

B2M RP – ATATAGAATTCATATTACATGTCTCGATCCCAC

Table 2-10. Thermocycler Settings For Amplification Of B2M Prior To Ligation Into pcDNA3.1(+)

Temp	Time	Cycles
98°C	0:30	1x
98°C	0:05	30x
64°C	0:15	
72°C	0:05	
72°C	5:00	1x

The amplified product and vector were then digested with EcoRV HF (Cat No. R3195S, NEB, Ipswich, MA, USA) and HindIII HF and re-run on an agarose gel to remove cut fragments as described in Chapter 2.8.3.1 – 2.8.3.2. After digestion, the pcDNA3.1(+) vector was also dephosphorylated and ligated at a 3:1 (insert:vector) ratio. The sample was then transformed into *E. Coli*, plated out, colonies picked and plasmid DNA extracted as described in Chapter 2.8.1.4 – 2.8.1.7. The vector was sent for sequencing to check for mutations within the B2M gene.

2.9 Cell Culture

2.9.1 Transfection Of CHO-FRT Cells With pcDNA5/B2M/HLA-B/MICA*009 Vector (Hygromycin Resistant)

CHO-FRT cells (Cat No. R75807, ThermoFisher, Waltham, MA, USA) were maintained in Ham's F-12 media (Cat No. B12-615F, Lonza, Basel, Switzerland) with 10% FCS (Cat No. 02-00-850, First-Link Ltd, Wolverhampton, UK), 1% penicillin/streptomycin (Cat No. P4333, Sigma Aldrich, St Louis, MI, USA) in 6-well plates and grown to 80% confluence. Prior to transfection, cells were washed with pre-warmed phosphate buffered

sale (PBS) and the media was substituted with 2 ml opti-mem low-serum media (Cat No. 31985062, ThermoFisher, Waltham, MA, USA).

Lipofectamine 2000 (Cat No. 11668019, ThermoFisher, Waltham, MA, USA) was used to transfect both pcDNA5/B2M/HLA-B/MICA*009 and pOG44 (Cat No. V600520, ThermoFisher, Waltham, MA, USA) into the CHO-FRT cells. This system utilises Flp-recombinase encoded in the pOG44 vector to ensure stable transfection of the GOI into the FRT site of the CHO-FRT cells, disrupting zeocin resistance and conferring hygromycin resistance. The pOG44 and pcDNA5/FRT vectors are designed to be transfected at a ratio of 9:1 respectively (Table 2-11).

After the DNA was added to the cells, the plates were incubated at 37°C, 5% CO_2 overnight. The following day, the cells were washed with pre-warmed PBS and the media replaced with Ham's F-12 with 10% FCS, 1% penicillin/streptomycin. Forty-eight hours after transfection 200ug/ml hygromycin (Cat No. H3274, Sigma-Aldrich, St Louis, MI, USA) selection was added to the media.

Table 2-11. Optimised Transfection Protocol For Lipofectamine 2000

Mock		Test	
15ul Lipofectamine 2000 + 150ul Opti-mem	9ug pOG44 plasmid DNA + 150ul Opti-mem	15ul Lipofectamine 2000 + 150ul Opti-mem	9ug pOG44 plasmid DNA + 1ug pcDNA5/B2M/HLA-B/MICA*009 plasmid DNA + 150ul Opti-mem
Incubate for 5 minutes at room temperature		Incubate for 5 minutes at room temperature	
Mix and incubate at room temperature for 20 minutes		Mix and incubate at room temperature for 20 minutes	
Add dropwise to labelled 6-well plate		Add dropwise to labelled 6-well plate	

2.9.2 Electroporation Of pcDNA3.1(+)-B2M And pcDNA3.1(+)-HLA-B Vectors Into CHO-MICA*009 Cells

The CHO-cells transfected with the triple construct pcDNA5/B2M/HLA-B/MICA*009 did not adequately express HLA-B (Discussed in Chapter 4). So, an alternative method was adopted. Rather than mounting three genes of interest into one large 8Kb vector, I used multiple vectors with different antibiotic resistance genes to introduce each gene to the CHO-cells. CHO-MICA*009 cells were already available from the Hayday laboratory, leaving B2M and the HLA-B constructs to be transfected. To do this I created 4 vectors, each expressing one GOI, as discussed in Chapters 2.8.3 & 2.8.4:

1. pcDNA3.1-B2M – Zeocin resistant
2. pcDNA3.1-HLA-**B*51** – neomycin resistant
3. pcDNA3.1-HLA-**B*52** – neomycin resistant
4. pcDNA3.1-HLA-**B*35** – neomycin resistant

The pcDNA3.1-B2M vectors was electroporated into the CHO-MICA*009 cells to ensure that all CHO-cells were able to express B2M and therefore HLA-B, as without B2M, HLA-B would not be expressed on the surface of CHO-cells. Once a stable population of CHO-MICA*009/B2M cells were created, three separate populations were created by transfecting each HLA-B construct. The CHO-MICA*009/B2M/HLA-B cells were then flow sorted and maintained in media with antibiotic selection.

CHO-MICA*009 cells were maintained in Ham's F-12 media with 10% FCS, 1% penicillin/streptomycin and 200ug/ml hygromycin in T175 flasks.

Prior to electroporation, media was removed and the cells were washed with pre-warmed PBS. Three millilitres of accutase (Cat No. A1110501, ThermoFisher, Waltham, MA, USA) was added and the flask was incubated at 37°C, 5% CO_2 for 5 minutes.

The cells were then collected and pelleted at 300g for 5 minutes. Three million cells were collected and washed with ice cold serum-free Ham's F-12 media. The cells were then resuspended in 250ul serum-free Ham's F-12 media and transferred to an ice cold 0.4cm electroporation cuvette (Cat No. 1652081, Bio-Rad, Hercules, CA, USA). Ten micrograms in 2ul of highly purified pcDNA3.1(+)/B2M (containing a Zeocin resistance gene) was transferred to the cuvette and immediately transferred to a Bio-Rad MicroPulse Electroporator (Cat No. 1652100, Bio-Rad, Hercules, CA, USA) and pulsed at 0.25kV, 950uF (high capacity and high range). The time correction was 50-60 seconds.

Following electroporation, cells were added to 4 ml of pre-warmed Ham's F-12 media with 10% FCS (no antibiotics) in a 6-well plate and allowed to recover for 24 hours before FACS sorting for a $MICA^+B2M^+$ population.

The $MICA^+B2M^+$ population was allowed to recover in antibiotic-free medium for 24 hours after FACS sorting before 100ug/ml zeocin and 200ug/ml hygromycin were added to the media. After 1 week of growth, pcDNA3.1(+)/HLA-B plasmids encoding neomycin-resistance were electroporated into the cell line using the same protocol. After resting, cells were FACS sorted for a $MICA^+B2M^+HLA\text{-}B^+$ population.

2.9.3 Electroporation Of HLA-B Constructs Into 722.221 HLA-Null Cells.

Despite good expression of the three constructs in the CHO-cells, they did not prove to be a good model for carrying out cytotoxicity assays (Discussed in Chapter 4). Thus, 722.221 (221) cells were used to transfect the three HLA-B constructs instead. 221-cells were maintained in Roswell Park Memorial Institute (RPMI) (+ L-glutamine) media (Cat No. BE12-702F, Lonza, Basel, Switzerland) with 10% FCS and 1% penicillin/streptomycin. Prior to electroporation, cells were agitated with a 5ml pipette to break up clumps and $3x10^6$ cells counted. 221-cells were washed three times in ice-cold PBS and centrifuged at 300g for 5 minutes per wash. The cells were then resuspended in 400ul ice-cold PBS and transferred into Bio-Rad electroporation cuvettes, which had been pre-chilled to 4°C. Ten micrograms in 2ul of pcDNA3.1(+)/HLA-B vector encoding zeocin resistance was added to the centre of the cuvette and kept at 4°C until electroporation.

Cuvettes were loaded into a Bio-Rad MicroPulser Electroporator and pulsed at 0.20kV, 975uF (Saunders et al., 2015). The time correction was 30-40 seconds. Electroporated cells were transferred to 6-well plates with RPMI and10% FCS (no antibiotics) for 24 hours and then flow sorted for HLA-B^+ cells. The same procedure was carried out for *HLA-B*51, B*52* and *B*35* constructs.

2.10 **Fluorescence Activated Cell Sorting (FACS)**

Flow cytometry was used to confirm expression of the transfected constructs on the surface of the target cell lines. Several panels were used throughout the project. FACS experiments were carried out on a FACS Canto II (BD Biosciences, San Jose, CA, USA)

and analysed with Flow-Jo software 8 &10 (TreeStar, Ashland, OR, USA) and a Novocyte 3000 (Acea Biosciences, San Diego, CA, USA) with Novoexpress software 1.2.5 (Acea Biosciences, San Diego, CA, USA).

2.10.1 Antibody And Fluorochrome Panels

2.10.1.1 Target Cells (CHO And 721.221-Cells)

A small, three-colour panel was used to identify MICA*009, B2M and HLA-B on the surface of CHO-cells. Only HLA was transfected into 221-cells, thus only one colour was used to ensure adequate expression (Table 2-12, Figure 2-4).

2.10.1.2 Peripheral Blood Mononuclear Cell Phenotyping And CD107a Expression

A larger eight colour panel was used to phenotype PBMC and carry out CD107a assays (Table 2-13, Figure 2-5).

Table 2-12. Antibodies Used For Extracellular Staining Of Target Cells.

Where more than one antibody is listed, the antibody listed first was used for functional assays. If poor staining was noted, then I tried a variety of antibodies to optimise the panel

Antibody	Marker	Fluorochrome	Manufacturer	Clone	Cat no.
Mouse Anti-human MICA/B	MICA	R-Phycoerythrin (PE)	BD Bioscience	6D4	558352
Mouse Anti-human B2M	B2M	Fluorescein Isothiocynate (FITC)	Biolegend	2M2	316304
Mouse Anti-human HLA-Class I	HLA-Class I	Allophycocyanin (APC)	Biolegend	W6/32	311409
		Allophycocyanin and cyanine dye (APC-Cy7)	Biolegend	W6/32	311429

Figure 2-4. Emission Spectra For Target Cell Fluorochrome Panel

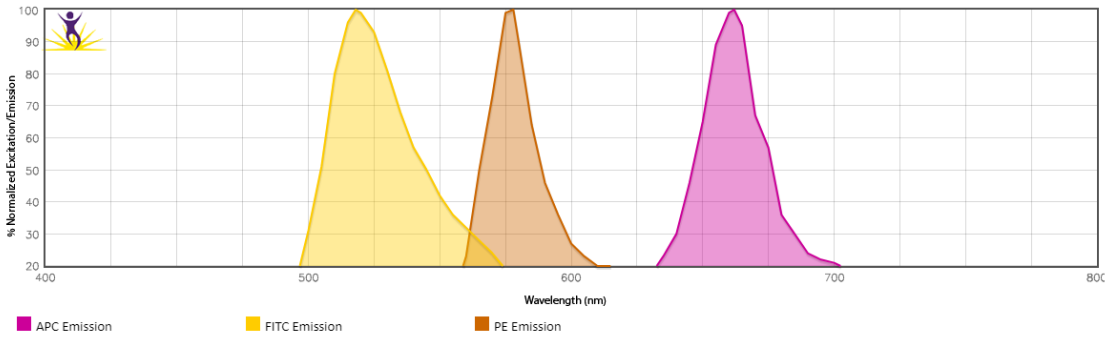


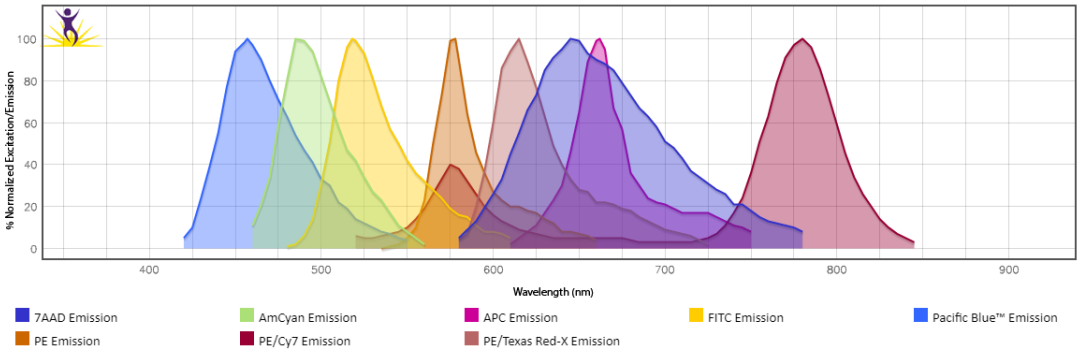
Table 2-13. Antibodies Used For Extracellular Staining Of PBMC

Where more than one antibody is listed, the antibody listed first was used for functional assays. If poor staining was noted, a variety of antibodies were used to optimise the assay.

Antibody	Marker	Fluorochrome	Manufacturer	Clone	Cat no.
Mouse Anti-human CD3	CD3	Pacific Blue (P. Blue)	Biolegend	HIT3a	344824
Mouse Anti-human CD8	CD8	FITC	Biolegend	SK1	300906
Human Anti-human CD56	CD56	PE	Miltenyi Biotec	AF12-7H3	130-100-622
		APC	Biolegend	HCD56	318309
Mouse Anti-human CD158e	DX9	Viogreen	Miltenyi Biotec	DX9	130-108-374
		APC	Biolegend	DX9	312715
Mouse Anti-human TCR γ/δ	γ/δ T	PE Cy7	Biolegend	B1	331222
Human Anti-human CD158e1/e2	CD158e1/e2	APC Vio 770	Miltenyi Biotec	REA 618	130-104-884
7-Aminoactinomycin D (7AAD)	7AAD	NA	Biolegend	NA	420403
Human Anti-human CD107a	CD107a	APC	Miltenyi Biotec	REA 792	130-112-000
		PE Vio 615	Miltenyi Biotec	REA 792	130-111-630
Mouse Anti-human CD107a		PE	BD Pharmingen	H4A3	555801

Figure 2-5. Emission Spectra From Peripheral Blood Mononuclear Cell Phenotyping Panel And CD107a Assay

Viogreen emits on the Amcyan channel and PE Vio615 emits on the PE Texas Red channel



2.10.2 Preparation Of Samples For Flow Cytometry

CHO-cells were first detached from the flask using an appropriate amount of accutase (1-5 ml) and incubated at 37°C for 5-10 minutes until floating freely. After this stage, all cell types were treated according to the same protocol. Cells were washed in FACS Buffer (PBS, 1mM EDTA, 2% FBS, 0.1% Sodium Azide (Cat No. S2002, Sigma Aldrich, St Louis, MI, USA)) and centrifuged at 800g for 5 minutes. The supernatant was then removed and the cells resuspended in 10 ml of FACS Buffer for counting. Approximately $1x10^5$ target cells (CHO or 221-cells) were stained to monitor extracellular expression of transfected constructs. The number of PBMC collected varied considerably between donors. Where possible, $1x10^7$ PBMC were used to examine the NK and CD8 T cell compartments.

Counted cells were then aliquoted into a 5ml polystyrene FACS tube. The tube was then centrifuged at 1,200g for 5 mins to pellet the cells. The supernatant was then removed and the tube placed on wet-ice.

An antibody cocktail was prepared using the manufacturers recommended doses of each antibody in 100ul FACS Buffer. Where relevant, fluorescence minus-one (FMO) antibody cocktails were prepared. The cells were resuspended in 100ul of antibody cocktail and vortexed to ensure even staining. The mix was then left to stain on wet-ice in the dark for 30 minutes. After 30 minutes, the cells were washed once with 1ml of FACS Buffer to remove any unbound antibody and resuspended in 300ul of FACS Buffer for analysis on a flow cytometer.

2.10.3 FACS Methodology

To ensure reproducible results, the staining protocol above was carried out on all samples. In order to allow experimental repeats to be carried out, PBMC were frozen in aliquots. Once thawed and stimulated (where relevant), PBMC were used in functional assays on the same day to avoid deterioration of the samples. Manual compensation for each fluorophore was carried out at the start of each experiment. If a fluorophore was substituted for a different colour, then compensation was redone.

2.10.4 CD107a Degranulation Assay

2.10.4.1 Stimulating Peripheral Blood Mononuclear Cells

Where possible, PBMC were stored in aliquots of $5x10^6$ to be used in degranulation assays with three experimental repeats. Prior to carrying out the assay, PBMC were thawed at 37°C and immediately washed with 10 ml PBS and re-pelleted at 800g for 5 mins. The cells were then stimulated with 1000U human IL-2 (Cat No. 130-097-742, Miltenyi

Biotech, Bergisch Gladbach, Germany) for 15-18hrs in RPMI + 10% FCS + 1% penicillin/streptomycin.

2.10.4.2 Counting Natural Killer Cells

After stimulation, PBMC were washed in PBS, counted using a haemocytometer and resuspended in culture media. A small aliquot of cells was then removed and the %NK cells determined using a flow cytometer ($CD3^{-}CD56^{+}$). An FMO for the flurophores attached to the anti-CD107a antibody was also run at this stage. The proportion of NK cells varied considerably. A target of $1x10^4$ NK cells per experiment was set, however this was not possible for many samples due to low numbers of circulating NK cells.

2.10.4.3 Preparing Target Cells

The target cell lines were counted at a 1:1 ratio of NK to target cells. Prior to plating, the target cell line was stained for surface expression of relevant markers and sorted using FACS Aria III (BD Biosystems, San Jose, CA, USA) if less 80%.

For the adherent CHO-cell line, $5x10^4$ cells were plated out 3 days prior to the assay and allowed to grow to near-confluence. To increase the number of events, each cell line was plated in triplicate. To ensure antibiotic selection did not interfere with the assay, antibiotics were removed from the culture media 1 day prior to the assay. On the day of the assay, the target cells were assessed to ensure near-confluence and washed with PBS. 100ul of antibiotic-free culture medium was then added and the cells incubated at 37°C, 5% CO_2.

The non-adherent 221-cells were removed to an antibiotic-free media the day before the assay and incubated at 37°C, 5% CO_2. On the day of the assay, 221-cells were counted and aliquoted into 5ml polystyrene FACS tubes in 100ul culture media.

2.10.4.4 Positive And Negative Controls

A positive control was set up consisting of PBMC in 200ul cell culture medium enriched with Phorbol 12-myristate 13-acetate (PMA) (Cat No. P8139, Sigma Aldrich, St Louis, MI, USA) to a final concentration of 2.5ug/ml and ionomycin (Cat No. IO634, Sigma Aldrich, St Louis, MI, USA) to a final concentration of 0.5ug/ml. A negative control was set up with PBMC and no target cells in 200ul cell culture media.

2.10.4.5 Setting Up The CD107a Assay

The PBMC were then aliquoted into either a 96 well plate (CHO-cells) or a FACS tube (221-cells) in 100ul culture media and 5ul of anti-CD107a. The samples were then incubated at 37°C in the dark. After 1 hour, 2ul of monensin (Golgistop, Cat No. 554724, BD Bioscience, San Jose, CA, USA) was added and the samples returned to the incubators for 4 more hours.

2.10.4.6 Preparing Samples For Staining

The cell culture media containing PBMC was gently removed from each well of the 96 well plate. PBMC from each sample run in triplicate were combined in a single FACS tube. The remaining adherent CHO-cells were then detached from the 96 well plate using

accutase and washed in PBS before being added to the PBMC. This step was taken to ensure any NK cells adherent to target cells were collected. The sample was then washed in FACS buffer and stained as above. 221-cells and PBMC were washed once in FACS buffer and stained.

2.11 Prospective Surveillance of Ophthalmic Manifesations of Behçet's Disease in the United Kingdom

2.11.1 Application To The British Ophthalmic Surveillance Unit

In order to carry out prospective surveillance of a rare ophthalmic manifestation in the UK, researchers can apply to use the infrastructure set up by the British Ophthalmic Surveillance Unit (BOSU), part of the Royal College of Ophthalmology (RCOphth).

This application process is formal and researchers submit Part 1 of the application form to BOSU detailing the population to be studied, the denominator, inclusion and exclusion criteria and projected numbers of patients.

2.11.2 Research Questions

- What is the incidence of BD related eye disease in the UK
- What is the clinical presentation and demographics
- What was the initial management
- What is the visual acuity at 3 months

2.11.3 Case Definition For Reporting Ophthalmologists

Patients must adhere to the International Study Group 1990 definition of BD and have at least one of the following: uveitis, retinal vein occlusions, retinal infiltrates, or hypopyon.

Clinicians should also report probable 'ocular-BD'. Whilst, this is not a common entity, it is known to ophthalmologists in the field and is defined by the presence of the ocular features of BD but without the systemic features to fulfil the International criteria mentioned above.

2.11.4 Expected Numbers And Rationale

Behçet's Disease is known to be most common across the ancient Silk Route and most of the epidemiological data has come from countries that are most affected. In 2001 Bang et al. reported a prevalence of 3.2/100,000, of these 50% (n=320) had eye disease(Bang et al., 2001). Data from Germany suggested that the prevalence of BD in 1997 was 1.68/100,000 with between 48%-66% (n=150-210) having BD related eye disease depending on the ethnic origins of the patient (Zouboulis et al., 1997). A smaller survey reported the incidence in northwest Spain at 0.66/100,000 with 30% having eye disease (n=34 over 5 years)(González-Gay et al., 2000).

To date, there has been no published data regarding the incidence of BD related eye disease in the UK.

I estimated the UK prevalence of BD to be 3/100,000. Over 30 years (based on 2,000 cases in UK as a whole) the incidence would be 1/1,000,000. I assumed that 20-50% have eye disease and would expect to survey 12-30 new cases of eye disease per year based on

the UK population of 64,000,000.

There will additionally be patients who present with eye disease confirming the diagnosis of BD, which I think will be an additional 13 cases, (25-43 cases/year in total).

There may also be cases reported where the patient presents with ‘ocular BD’ (eye involvement but not fulfilling the international criteria mentioned above). I anticipate this to be around 5 cases/year. Therefore, the total expected cases of BD related eye disease is 30-48 per year.

2.11.5 Funding And Sponsorship

The BOSU costs of this project were funded by Prof. Miles Stanford. Moorfields Eye Hospital were the sponsors of the project.

2.11.6 Ethics And Health Research Authority Approval

This project was granted ethical approval from North London Research Ethics Committee (IRAS 199980) in December 2015. A minor amendment was added in April 2016, which changed the data collection field ‘Month/Year of Birth’ to ‘Age’. This was done on request of the Health Research Authority and Sponsor to reduce the chances of patients being identified.

2.11.7 Consent And The Data Collection Tool

All information collected in BOSU studies is non-identifiable, thus patients are not routinely consented to take part in surveillance studies. The data collection tool is peer reviewed by the BOSU committee and changes are made in conjunction with the researchers. The final data collection tool is submitted to the Health Research Authority and the Research Ethics Committee to obtain approval.

2.11.8 Methodology

Each month, all consultant and associate specialist ophthalmologists in the UK receive a Yellow Card with up to five ophthalmic conditions to report along with a self addressed envelope to the RCOphth. 'New Ophthalmic Involvement in Behçet's Disease' was included on the Yellow Card one from November 2016-November 2017. When a new case is reported to BOSU, I received an email to notify me who had reported the case and where they worked.

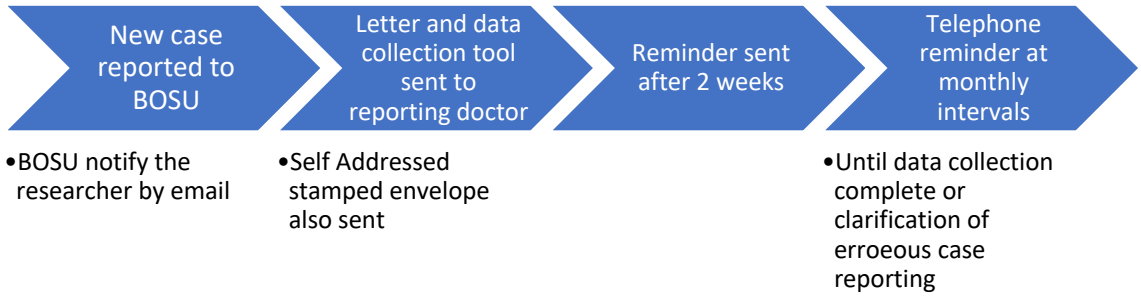
I then posted a letter describing the study and a data collection tool (Appendix 5 - Questionnaire for Reporting Clinicians – Prospective Surveillance of Ophthalmic Manifestations of Behçet's Disease.), along with a self-addressed envelope. If there was no reply after two weeks a reminder was posted. After one month, if there was no response, I would telephone the individual and remind them verbally. Monthly telephone reminders would continue until either a response was received, or I was informed that the case was reported in error (Figure 2-6).

2.11.9 Data Collection

Data was transcribed from the retuned paper questionnaires into a Microsoft Excel 2016 spreadsheet (Microsoft Corporation, Redmond, WA, USA). While patient identifiable information was not collected, details about patient demographics were collected. This included age at presentation, ethnicity, gender and the first half of the patients' postcode. Details of laterality was collected along with the specific dates the patient developed systemic signs of BD.

Figure 2-6 . Timeline For Sending Out Questionnaires To Reporting Clinicians For Prospective Surveillance Study.

In order to reduce the number of missed or incomplete cases, it is important to send a number of reminders to reporting clinicians. In this project, I sent biweekly reminders by mail and then followed up with telephone calls. BOSU – British Ophthalmic Surveillance Unit



I collected information regarding systemic involvement and pre-existing therapy for BD. Visual acuity can be measured in a number of ways and was collected in either Logarithm of the Minimal Angle of Resolution (LogMAR), or Snellen format and converted to

LogMAR-equivalents for analysis. Any pre-existing reason for poor vision was also documented. Reporting clinicians were asked to identify the nature of ophthalmic involvement and the first line treatment given.

Finally, I collected information regarding the referring unit and where the patient had been referred to or from. This was to allow identification of duplicate reporting from different centres.

2.11.10 Analysis

The purpose of this study was to establish the incidence of ophthalmic manifestations in BD. Incidence was calculated by dividing the number of correctly identified cases by the population of the United Kingdom in 2017. Census data was obtained from the Office of National Statistics (ONS). 95% CI of the incidence was calculated using the Wilson Method and was calculated using Ausvet statistical package: Sergeant, ESG, 2019. Epitools epidemiological calculators. Ausvet. www.epitools.ausvet.com.au**.** Differences in visual acuity between groups was assessed using a two tailed Analysis of Variance (ANOVA) with $P<0.05$ being considered significant.

2.12 Statistical Analysis

Statistical analyses were carried out my myself. Immunogenetic data was imported into Microsoft Excel and manipulated within the spreadsheet. Fisher's Exact Test was used to calculate *P* values for immunogenetic data. A Microsoft Excel plugin was used to carry out Fisher's Exact Test within Microsoft Excel (http://www.obertfamily.com/software/fisherexact.html). Odds Ratios (OR) were

calculated in Microsoft Excel by dividing the odds of possessing an allele in the disease group by the odds in the control group. Standard errors were calculated using a standard formula and 95% confidence intervals were constructed for each OR. When n=0, the Haldane correction was used to approximate an OR(Haldane, 1945), this is a method of adding 0.5 to. 0 field and subtracting 0.5 from the total in order to avoid an error in the calculation.

P values were corrected for multiple testing using the Bonferroni-Dunn Correction to reduce the risk of Type 1 error, this was carried out using GraphPad Prism Version 7 for Macintosh (GraphPad Software, La Jolla, CA, USA). Analysis of variance (ANOVA) was used to compare three or more group means and was used to determine whether specific ophthalmic manifestations of BD could have caused patients to lose more vision. Graphs were constructed in GraphPad Prism and Microsoft Excel. R^2 correlations were carried out in the functional analyses to determine whether two variables were correlated or not. Correlation was carried out in GraphPad Prism. χ^2 was used to determine whether there was a difference in the expected and observed frequencies of alleles and was also carried out in GraphPad Prism.

Post-hoc power was calculated in https://clincalc.com/stats/Power.aspx to allow an approximation of the risk of Type II errors to inform the discussion. In all cases an α of 0.05 was assumed for post-hoc calculations. Post-hoc power was only calculated for significant results.

3 Confirming Known Genetic Risk Factors In A UK Cohort Of Individuals With Behçet's Disease

3.1 The UK Behçet's Disease Cohort

In 2013, the BD Centres of Excellence were commissioned by the National Health Service (NHS) England. These centres were created to give patients with a possible/probable/definite diagnosis of BD access to a multidisciplinary team of doctors, nurses, psychologists and support workers with a specialist interest in the condition.
In addition to providing up-to-date and thorough clinical care, the centres have access to newer biologic therapies and are funded directly from NHS England. They also offer a unique platform for conducting research as patients are well-characterised and highly motivated to participate.

There are now over 2000 patients registered across three sites in the UK. In order to investigate whether previously reported genetic risk factors are applicable to a UK cohort, I collected DNA from 267 BD patients and 445 HCs and analysed a number of immunogenetic loci.

3.2 Behçet's Disease And Healthy Control Demographics

Patients for this project were recruited sequentially from the London clinic between 01/2006-2/2015, irrespective of ethnicity or disease severity. Demographically matched HC were recruited from unrelated friends, partners and spouses of patients.

Inclusion criteria:

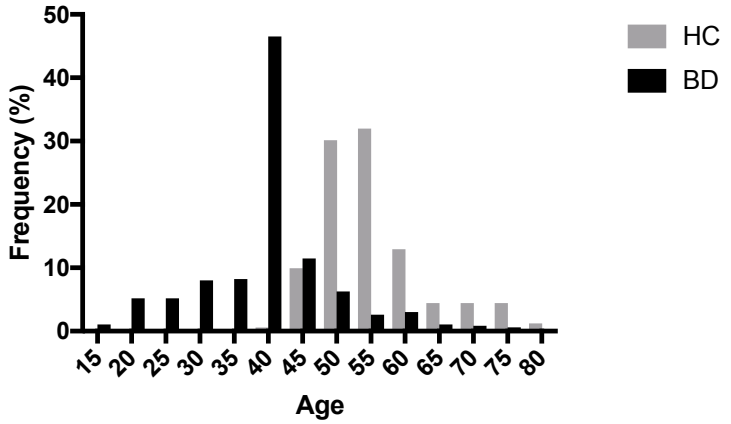
- Confirmed BD as per ISG 1990 criteria (ISG, 1990)
- Over the age of 16 years

3.3 Age

All individuals over the age of 16, fulfilling the inclusion criteria were eligible to be included in the study. Healthy controls were deliberately selected to be older than the cases to avoid recruiting individuals with unmasked inflammatory disease. Behçet's Disease usually presents before the age of fifty, so wherever possible HCs were selected to be older than 40 years. As expected, the two populations were significantly different with respect to age (P=0.0001). The median age of the BD group was 41 years (interquartile (IQ) range 36-43 years). The median age of the HC group was 54 years (IQ range 52-58 years). Neither group was normally distributed (P<0.0001). Figure 3-1 is a histogram, illustrating the range of ages in both groups.

Figure 3-1. Histogram Illustrating The Ages Of The Behçet's Disease And Healthy Control Groups At The Time Of DNA Extraction.

The median age of the HC group was 13 years older than the BD group. HC – Healthy Control, BD – Behçet's Disease.



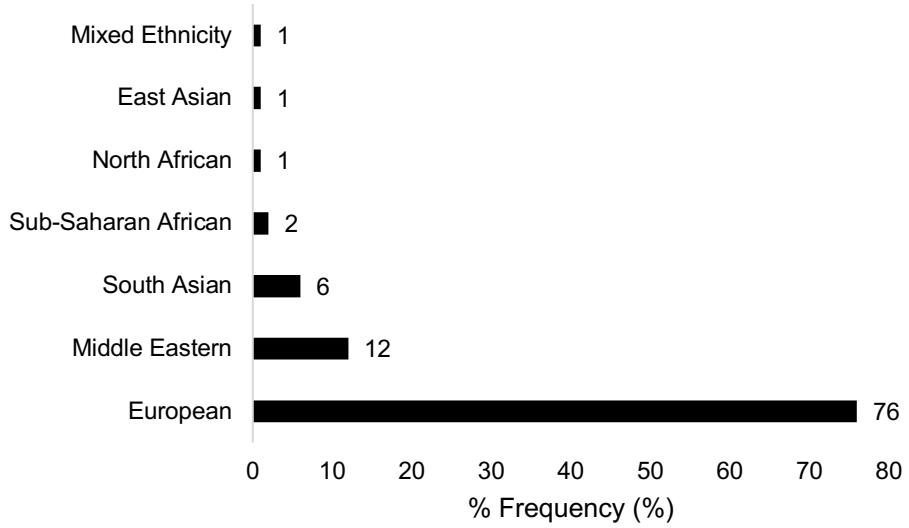
3.4 Ethnicity

A full family history was taken at the time of consenting to accurately capture an individual's ethnicity. These were then condensed into United Nations ethno-geographic regions (https://unstats.un.org/unsd/methodology/m49/overview/), or identified as 'mixed ethnicity' where this was not possible.

Figure 3-2 shows the ethnicity of patients and healthy controls recruited in the study. As can be seen, 76% of individuals were European. These were patients who claimed that all their antecedents for 3 generations were from Europe. Twelve per cent of participants claimed to be of Middle-Eastern ancestry and 6% from South Asia. Table 3-1 shows the breakdown of ethno-geographic regions by country of birth for each participant.

Figure 3-2. Self-Reported Ancestry Of Participants By UN Ethno-Geographic Regions

The majority of participants were of European ancestry. Percentages are shown at the end of each bar.



3.5 Clinical Manifestations Of Behçet's Disease

Behçet's Disease is a clinical diagnosis. There are two widely used diagnostic criteria. The ISG 1990 criteria (ISG, 1990), was designed principally as a research tool, whereas the ICBD criteria (International Team for the Revision of the International Criteria for Behçet's, 2014) was designed to aid clinical diagnosis. All individuals recruited for this study fulfilled the ISG 1990 criteria. Table 3-2 shows a breakdown of clinical manifestations documented for each patient over their disease-course.

In 2008, Mahr et al. published an epidemiological analysis BD in a Parisian county (Seine-Saint-Denis) (Mahr et al., 2008). The investigators collected data using health-registry databases and questionnaires given to care-providers. Patients with BD were then subdivided by ethnicity based on the country of origin of their grandparents, resulting in five groups (European, Asian (including Turkish), Sub-Saharan African, North African, Non-continental French and Other). I used the data gathered from the 79 individuals in this study to compare the manifestations of BD in the UK cohort and found no difference between the percentage of individuals with different disease manifestations (P=0.5582).

Table 3-1. Participant Ethno-Geographic Regions By Country Of Birth

Europe	Belgium
	France
	Germany
	Holland
	Ireland
	Sweden
	United Kingdom
	Greece
	Italy
	Spain
Middle East	Egypt
	Israel
	Jordan
	Palestine
	Syria
	Turkey
South Asia	Afghanistan
	Bangladesh

	India
	Pakistan
Sub-Saharan Africa	Nigeria
	Uganda
East Asia	China
	Japan
	Korea
	Vietnam
North Africa	Morocco

Table 3-2. Clinical Manifestations Of Behçet's Disease In The UK Cohort Compared To Another European And Non-European Cohort.

Column in bold represents the UK cohort described in this project.

	United Kingdom %	European % (Mahr et al., 2008)	Non-European % (Mahr et al., 2008)
Recurrent oral ulceration	**100**	100	100
Cutaneous manifestations	**81**	95	88
Genital ulceration	**78**	79	80
Arthropathy/Arthritis	**60**	58	60
Ocular manifestations	**30**	58	48
Vascular manifestations	**16**	37	28
Neurological involvement	**15**	5	12
Gastrointestinal	**5**	Not Reported	Not Reported

Of note, the frequency of eye disease in both the European and non-European cohorts were higher than in the UK. There are a number of possibilities that could explain this finding:

1. Ophthalmic disease in the UK cohort was reported by a uveitis specialist who saw all the patients and only documented BD-related eye disease. Mahr et al. extracted data from registries which are likely to have less granular detail and may include ophthalmic problems that are not BD-related.
2. Individuals from the Middle-East and Southern Europe have been noted to have a higher incidence of ophthalmic involvement in BD. There is little reliable data to compare the incidence of BD-related eye disease in Northern Europe.

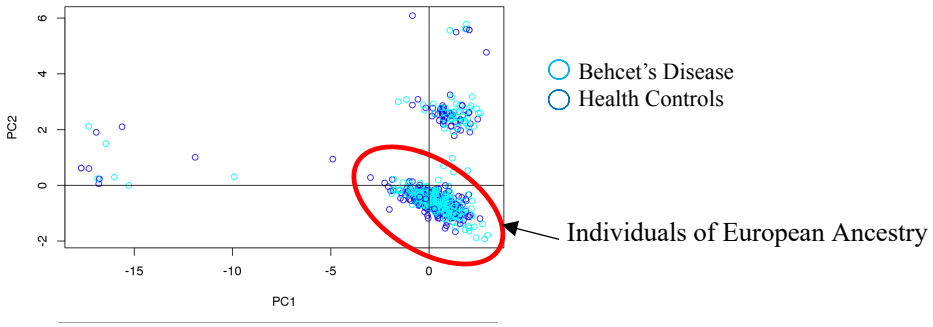
3.6 European Ancestry Informative Markers

Study of *HLA* and its associated genes is complicated by huge variation within the region. In addition, there is substantial geographic variation. In order to conduct robust studies of specific *HLA* associations, it is useful to ensure the study population is defined and that the control group is matched. To try and reduce the noise caused by population admixture, In collaboration with the Genome Centre at QMUL, we applied a set of ancestry informative markers (AIMs) specific for European populations to the sequencing panel. Imperfect matching between cases and controls can lead to spurious associations, or failure to detect true associations (Marchini et al., 2004). In 1978, Menozzi et al. described the use of principle component analysis (PCA) in human genetics (Menozzi et al., 1978). Highly dimensional data can be summarised by PCA to capture the variables that best describe the data set, allowing visualisation of allele frequency differences amongst populations. It is possible to correlate principle components of data with meaningful geographic axes. Shriver et al. proposed that certain markers with distinct frequency differences across populations may be highly informative for assigning ancestry (Shriver et al., 2003). These markers are referred to as AIMs. A small number of AIMs may be used to perform population clustering. We used a panel of 25 AIMs designed to identify individuals from Europe in order to exclude individuals in the data set who may have had ancestry elsewhere in the world. Amplicons for each AIM were designed, amplified and sequenced in simultaneous runs to other immunogenetic loci. The sequences were then aligned and SNPs called. Principle component analysis was used to delineate the spread of AIMs. Only those individuals with SNPs equating to Europe were used in the European subgroup (Figure 3-3). This work was carried out by Ian Donaldson at the QMUL Genome Centre.

After quality control, there were 197 individuals (73% of the total BD cohort) with BD and 335 HCs (75% of the total HC cohort) in the European subgroup, enabling us to match BD and HC individuals at an ancestral level.

Figure 3-3. Principle Component Analysis Of European Ancestry Informative Markers

Using PCA, the AIMs were clustered and individuals not of European ancestry were excluded from the subgroup analysis



3.7 Associations Between *HLA-A & B* And Behçet's Disease In The United Kingdom Cohort

3.7.1 *HLA-A & B* Allele Frequencies

A total of 712 individuals were recruited, made up of 267 patients with BD and 445 demographically matched healthy controls (HC). *HLA-A* was typed at medium-high resolution on a Luminex platform. *HLA-B* and *MICA* alleles were determined using sequence-based typing at high-resolution (Appendices 2 and 3) and ambiguous calls were confirmed on a Luminex platform or via SSP (Appendix 1). After quality control, *HLA-A* alleles were obtained from 260 (97.38%) cases and 426 (95.73%) controls (Figure 3-4), *HLA-B* alleles were obtained from 266 (99.63%) cases and 443 (99.55%) controls (Figure 3-5).

Relevant *HLA* associations are shown in Table 3-3. Within the UK cohort, *HLA-B*51* was associated with BD (P=1.52x10^{-8} Pc=4.42x10^{-7}, Odds Ratio (OR)=2.86, 95% Confidence

Interval (CI) 1.98-4.13). Smaller risk effects were also identified for *HLA-B*18,* but lost significance after correcting for multiple testing (*P*=0.0054, *Pc*=0.1236, OR=2.25, 95% CI 1.29-3.72), *HLA-A*25 (P*=0.0121, *Pc*=0.2427, OR=2.51, 95% CI 1.17 - 5.41) and *HLA-A*26* (*P*=0.0291, *Pc*=0.4634, OR=1.76, 95% CI 1.00 - 3.10). Protective effects were found from *HLA-B*58* (*P*=0.0017, *Pc*=0.0489, OR=0.09, 95% CI 0.01-0.64), *HLA-B*15* (*P*=0.0065, *Pc*=0.1558, OR 0.48, 95% CI 0.29-0.82), *HLA-B*44* (*P*=0.0485, *Pc*=0.4586, OR=0.71, 95% CI 0.51-0.99) and *HLA-A*33* (*P*=0.0131, *Pc*=0.2645, OR=0.45, 95% CI 0.22 - 0.92), however the latter three alleles did not survive correction for multiple testing.

Figure 3-4. *HLA-A* Allele Frequencies >1% Within The United Kingdom Cohort
There were no HLA-A alleles that were associated with BD, after correcting for multiple testing

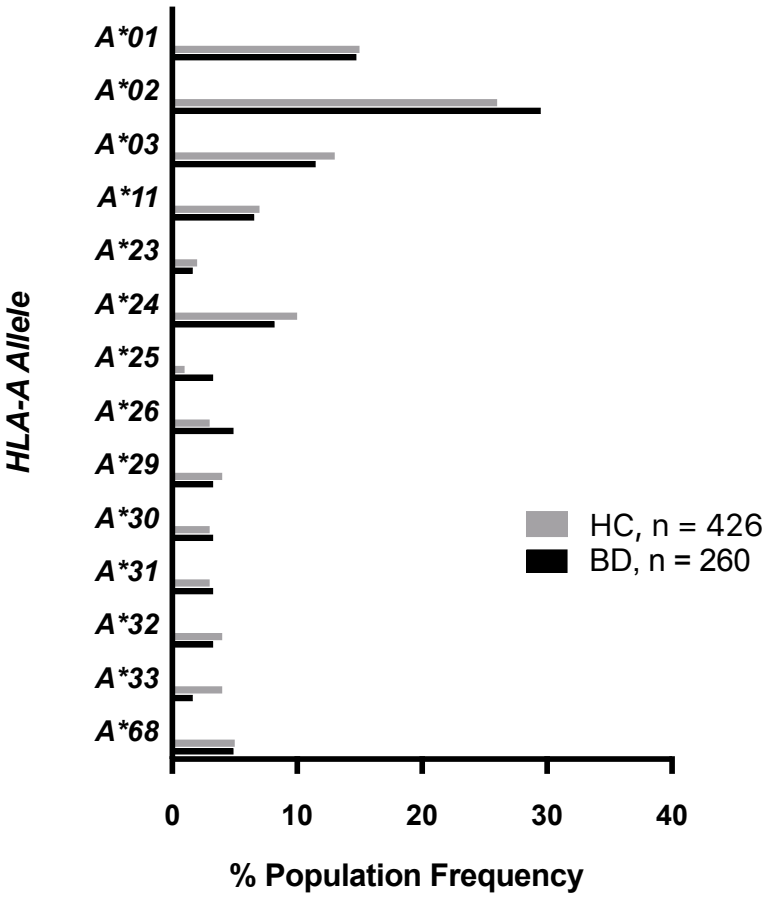


Figure 3-5. *HLA-B* Allele Frequencies > 1% Within The United Kingdom Cohort
*HLA-B*51 was associated with BD (*** indicate P<0.0001) after correcting for multiple testing.*

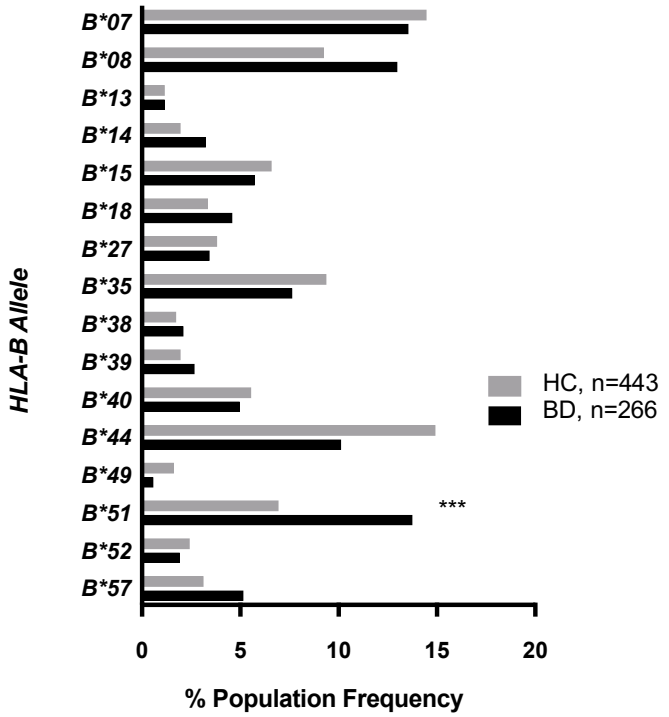


Table 3-3. *HLA-A* And *-B* Associations In The United Kingdom Cohort.
*P was considered significant if <0.05. Pc – Bonferroni-Dunn correction for multiple testing, P and Pc in **Bold** are significant after correction for multiple testing. HLA – Human Leukocyte Antigen, UK – United Kingdom, HC – Healthy Control, BD – Behçet's Disease, OR – Odds Ratio, 95% CI – 95% Confidence Interval*

HLA Type	UK Cohort (HC n=445, BD n=267)							
	HC (n)	**HC (%)**	**BD (n)**	**BD (%)**	***P***	***Pc***	**OR**	**95% CI**
Increased Risk								
*HLA-B*51*	52	6.06	80	15.56	**4.34×10^{-5}**	**0.0009**	2.13	1.49-3.06
*HLA-B*18*	23	2.68	30	5.84	0.0054	0.1236	2.25	1.29-3.92
*HLA-A*25*	11	1.2	17	3.1	0.0121	0.2427	2.51	1.17-5.41
*HLA-A*26*	24	3.2	26	5.1	0.0291	0.4634	1.76	1.00-3.10
Reduced Risk								
*HLA-B*58*	19	2.21	1	0.19	**0.0017**	**0.0489**	0.09	0.01-0.64
*HLA-B*15*	63	7.34	19	3.70	0.0065	0.1558	0.48	0.29-0.82
*HLA-B*44*	126	14.69	56	10.89	0.0485	0.4586	0.71	0.51-0.99
*HLA-A*33*	36	4.3	10	2.3	0.0131	0.2645	0.45	0.22-0.92

3.7.2 *HLA-B* Associations In The *HLA-B*51*$^{-}$ Individuals In The United Kingdom Cohort

*HLA-B*51*$^{-}$ individuals were then analysed to see whether other *HLA-B* associations became relevant. In this analysis, the *HLA-B*18* association strengthened and became significant after correcting for multiple testing (*P*=0.0018, *Pc*=0.0458, OR=2.47, 95% CI 1.42-4.32).

3.7.3 Presence/Absence And Gene-Dosage Of Bw4 Bearing Alleles In The United Kingdom Cohort

Alleles were identified as coding for Bw4 or Bw6 based on current databases published by the European Bioinformatics Institute. Presence or absence of Bw4 was analysed for each individual and gene-dosage of Bw4-bearing alleles was calculated (i.e. an individual may have a maximum of four Bw4 bearing-alleles if both alleles of *HLA-A* & *B* encode an *HLA* allotype with the Bw4 epitope such as *HLA-A*23/A*24* and *HLA-B*51/B*44*). I found no disease association between presence/absence or gene dosage of Bw4-bearing alleles in the UK cohort (Figure 3-6).

Figure 3-6. Gene Dosage Of Bw4 In The United Kingdom Cohort.

There was no difference in the presence/absence or dosage of Bw4 between BD and HC.

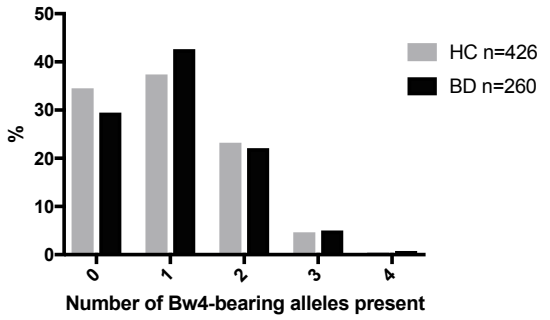


Table 3-4. *HLA-A* And *-B* Associations In The European Subgroup.

Those in bold signify significant (P<0.05) results. Bonferroni was not applied the subgroup testing due to its punitive effects on power. HLA – Human Leukocyte Antigen, UK – United Kingdom, HC – Healthy Control, BD – Behçet's Disease, OR – Odds Ratio, 95% CI – 95% Confidence Interval

HLA-type	**European ancestry subgroup (HC n = 335, BD n = 198)**						
	HC (n)	**HC (%)**	**BD (n)**	**BD (%)**	***P***	**OR**	**95% CI**
Increased Risk							
*HLA-B*51*	47	7.01	46	11.62	**0.0131**	1.74	1.14-2.67
Reduced Risk							
*HLA-B*58*	15	2.24	2	0.51	**0.0398**	0.22	0.05-0.97

3.8 Associations Between *HLA-A* & *-B* And Behçet's Disease In The European Subgroup

I applied the AIMs to the UK cohort to create a European subgroup of BD and HCs. The European subgroup comprised 532 individuals of whom 198 had BD (37.2%) and 334 were HC (62.8%). *HLA-A* alleles were available for 192 BD (97.5%) and 321 HC (95.9%). *HLA-B* alleles were available for 197 BD (100%) and 333 HC (99.4%).

3.8.1 *HLA-A* & *-B* Allele Frequencies In The European Subgroup

In the European subgroup, *HLA-B*51* maintained an association with BD (*P*=0.0131, *Pc*=Not Applied (NA), OR 1.74, 95% CI 1.14-2.67) and *HLA-B*58* conferred protection (*P*=0.0398, *Pc*=NA, OR=0.22, 95% CI 0.05-0.97) (Table 3-4). No other associations were identified.

3.8.2 *HLA-B* Associations In *HLA-B*51*$^{-}$ Individuals In The European Subgroup

I then conditioned on the *HLA-B*51*$^{-}$ individuals in the European subgroup. *HLA-B*58* retained its protective effects in this group (*P*=0.0011, *Pc*=NA, OR=0.06, 95% CI 0.01-0.94), but no risk-alleles were identified.

3.8.3 Presence/Absence And Gene-Dosage Of Bw4 Bearing Alleles In The European Subgroup

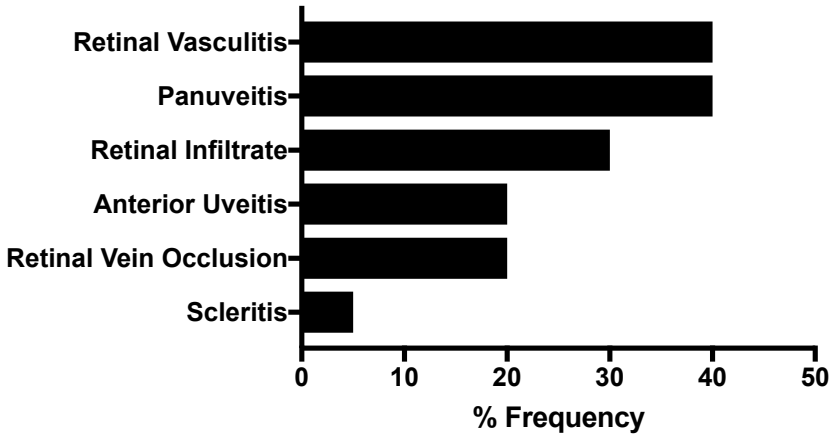
I repeated the Bw4 presence/absence and gene dosage analysis in the European subgroup, but there were no associations between Bw4 and BD identified.

3.9 *HLA-A* & *-B* Associations In Patients With Ophthalmic Manifestations Of Behçet's Disease

There is evidence to suggest that *HLA-B*51* is associated with ophthalmic manifestations of BD (Gul et al., 2001). To investigate this in our cohort, I stratified the disease group into those with mucocutaneous disease (MC) and those with ophthalmic involvement. I

identified 80 individuals in the cohort with ophthalmic manifestations of BD. *HLA-B*51* was strongly associated with ocular involvement in our cohort (P=3.71x10^{-8}, Pc=NA, OR=4.17, 95% CI 2.58-6.74), with an odds ratio 1.6x higher than in MC disease alone (P=1.78x10^{-5}, Pc=NA, OR 2.57, 95% CI 1.70-3.91). Figure 3-7 shows the breakdown of ocular manifestations in BD found in the UK cohort. As can be seen, retinal vasculitis and panuveitis were the most common manifestations, occurring in 40% of individuals. Retinal infiltrates were identified in 30% and anterior uveitis and retinal vein occlusions were reported in 20%. Scleritis is unusual in BD and not typically thought to be an associated pathological finding, but was present in 5% of individuals.

Figure 3-7. Ocular Manifestations of Behçet's Disease in the United Kingdom Cohort (n=80)



3.10 *MICA* Allele Frequencies In United Kingdom Cohort

MICA allele level sequencing is discussed in materials and methods. Allele frequencies were calculated for the UK cohort and the European subgroup. After quality control, *MICA* alleles were available for 431 HC (96.9%) and 262 BD (98.1%). Figure 3-8 shows the breakdown of allele frequencies in the UK cohort.

There was a suggestion that *MICA*009* (A6) contributed to the risk of developing BD (*P*=0.0002, *Pc*=0.0045, OR=1.80, 95% CI 1.31-2.47). There was also a small effect from *MICA*019* (A5) (*P*=0.0383 *Pc*=0.4434, OR 1.72, 95% CI 1.05-2.81). Allele frequencies can be seen in Table 3-5.

Five different TM repeats were found in the HC and BD population (A4, A5, A5.1, A6, A9). However, no specific TM repeat was associated with BD. Analysis of the frequency of TM repeats can be found in

Table 3-6. The *MICA* TM repeat frequencies were compared against reported frequencies in European cohorts (http://www.allelefrequencies.net/mic6001a.asp) to test HWE and were not found to deviate substantially (χ^2 = 3.468, df 4, *P*=0.4812).

Figure 3-8. *MICA* Allele Frequencies In The UK Cohort.

*MICA*009 (A6) is associated with BD (** = P<0.001) after correction for multiple testing.*

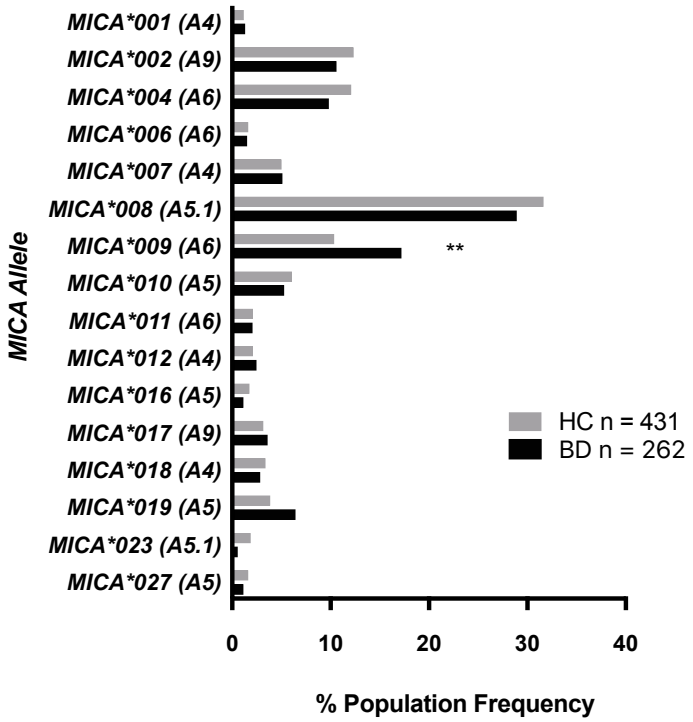


Table 3-5. *MICA* Allele Frequencies In The UK Cohort (Ordered By Effect Size)

*There is an association between MICA*009 (A6) and BD. HC – Healthy Control, BD – Behçet's Disease, OR – Odds Ratio, 95% CI – 95% Confidence intervals*

MICA	**TM Repeat**	**UK Cohort (HC n = 431, BD n = 262)**							
		HC (n)	**HC (%)**	**BD (n)**	**BD (%)**	***P***	***Pc***	**OR**	**95% CI**
Increased Risk									
*MICA*009*	A6	89	10.35	91	17.20	**0.0003**	**0.0045**	1.80	1.31-2.47
*MICA*019*	A5	33	3.84	34	6.43	**0.0383**	0.4434	1.72	1.05-2.81
*MICA*012*	A4	18	2.09	13	2.46	0.7096	0.9997	1.18	0.57-2.43
*MICA*017*	A9	27	3.14	19	3.59	0.6462	0.9997	1.15	0.63-2.09
*MICA*001*	A4	10	1.16	7	1.32	0.8053	0.9997	1.14	0.43-3.01
*MICA*008*	A5.1	272	31.63	153	28.92	0.3082	0.9880	0.88	0.69-1.11
*MICA*007*	A4	43	5.00	27	5.10	1.0000	1.0000	1.02	0.62-1.67
Reduced Risk									
*MICA*011*	A6	18	2.09	11	2.08	1.0000	1.0000	0.99	0.47-2.12
*MICA*006*	A6	14	1.63	8	1.51	1.0000	1.0000	0.93	0.39-2.23
*MICA*010*	A5	52	6.05	28	5.29	0.6356	0.9997	0.87	0.54-1.39
*MICA*002*	A9	106	12.33	56	10.59	0.3447	0.9904	0.84	0.6-1.19
*MICA*018*	A4	29	3.37	15	2.84	0.6384	0.9997	0.84	0.44-1.57
*MICA*027*	A5	14	1.63	6	1.13	0.4978	0.9990	0.69	0.26-1.82
*MICA*004*	A6	104	12.09	52	9.83	0.2206	0.9608	0.79	0.56-1.13
*MICA*016*	A5	15	1.74	6	1.13	0.4981	0.9990	0.65	0.25-1.68
*MICA*023*	A5.1	16	1.86	3	0.57	0.0554	0.5499	0.30	0.09-1.04

Table 3-6. Frequency Of MICA Transmembrane Repeats In The UK Cohort.

There is no association between TM repeats and BD. TM – Transmembrane, HC – Healthy Control, BD – Behçet's Disease

TM Repeat	**UK Cohort (HC n = 431, BD n = 262)**				
	HC (n)	**HC%**	**BD (n)**	**BD%**	***P***
A4	100	11.63	62	11.72	1.0000
A5	114	13.26	74	13.99	0.7503
A5.1	288	33.49	156	29.49	0.1212
A6	225	26.16	162	30.62	0.0706
A9	133	15.47	75	14.18	0.5412

Table 3-7. *HLA-B* Allele Frequencies In The *MICA*009*$^{+}$, *HLA-B*51*$^{-}$ Population.

*Due to the strong LD that exists between MICA*009 and HLA-B*51, I investigated associations in the MICA*009*$^{+}$*, HLA-B*51*$^{-}$ *population. There was a protective effect found between HLA-B*52 and MICA*009 in the HC group. HC – Healthy Control, BD – Behçet's Disease, LD – Linkage Disequilibrium, Other – HLA-B Alleles with a frequency of less than 5%.*

***HLA-B* allele**	***MICA*009*$^{+}$, *HLA-B*51*$^{-}$ individuals *in the UK Cohort (HC n = 82, BD n = 98)***							
	HC (n)	**HC (%)**	**BD (n)**	**BD (%)**	***P***	***Pc***	**OR**	**95% CI**
*B*52*	20	24	5	5	**0.0003**	**0.0028**	0.21	0.07-0.59
*B*07*	15	18	25	26	0.2827	0.7353	1.39	0.68-2.87
*B*35*	12	15	6	6	0.0799	0.3934	0.42	0.15-1.17
*B*08*	7	9	16	16	0.1778	0.6242	1.91	0.75-4.9
*B*44*	7	9	12	12	0.4731	0.8401	1.43	0.54-3.83
*B*27*	2	2	5	5	0.4572	0.8401	2.09	0.4-11.08
*B*40*	2	2	10	10	0.0679	0.3886	4.18	0.89-19.67
Other	17	21	19	19	0.8532	0.8532	0.94	0.45-1.94

3.11 *MICA* Allele Frequencies In European Subgroup

The European AIMs were then applied to the *MICA* data to create a European subgroup. After quality control, *MICA* sequences were available for 181 BD (91.7%) and 318 HC (95.4%). A planned-comparison test confirmed that the associations found in the UK cohort were maintained within the European subgroup, but with a smaller effect size. *MICA*009* (A6) (*P*=0.0013, *Pc*=NA, OR=1.85, 95% CI 1.28-2.68) contributed to the risk of developing BD. There was no association between any TM repeat in the European subgroup.

3.12 *MICA* Allele Frequencies In *HLA-B*51*$^{-}$ Individuals

Linkage disequilibrium (LD) between *HLA-B*51* and *MICA*009* (A6) was calculated in the UK cohort and European subgroup (*D'*=0.88 and 0.82 respectively). Given the strong LD between these regions, I was curious to analyse the *MICA* allele frequency in the *HLA-B*51*$^{-}$ population. I found that *MICA*009* (A6) maintained its disease association (*P*=0.0001, *Pc*=NA, OR=2.32, 95% CI 1.50-3.58). To ascertain whether this *MICA* association is independent of *HLA* or in LD with another *HLA-B* locus, the *HLA* alleles carried by individuals in this group were explored (Table 3-7). Protective effects from carriage of HLA-B*52 and MICA*009 (*P*=0.0003, *Pc*=0.0028, OR=0.21, 95% CI 0.07-0.59) were found. This finding is not surprising as *HLA-B*51* and *B*52* are closely related and there are previous reports of LD between *HLA-B*52* and MICA*009 despite its protective effects in BD (Eyerci et al., 2018). *MICA* transmembrane polymorphisms were then analysed for the *HLA-B*51*$^{-}$ population, but no specific TM repeat was associated with BD.

3.13 *MICA* Allele Frequencies In Ophthalmic Behçet's Disease

The group of BD cases with ophthalmic involvement was then analysed to ascertain whether there were any *MICA* associations specific to that group. I found a *MICA*009* (A6) association (*P*=0.0029, *Pc*=NA, OR=2.03, 95% CI 1.29-3.20) in keeping with the trend from the larger cohort, but no other significant results. There was no specific TM repeat associated with ophthalmic disease.

3.14 Discussion Of Findings

This study aimed to identify whether previously reported genetic susceptibility loci were applicable to a UK cohort of individuals with BD, a group which is not well described in the literature. 76% of individuals with BD in the UK claim to be of European ancestry. Using a panel of AIMs, 73% of individuals had AIMs that were consistent with a European lineage; a finding that closely correlated with the self-reported ethnicity from participants.

*HLA-B*51* was associated with BD with an OR of 2.13. The data was well powered to calculate this association with a post-hoc power of 98%. When the data was filtered to only examine those of European ancestry, *HLA-B*51* remained significant, but the OR reduced to 1.74 (Table 3-3 and Table 3-4) and the post-hoc power reduced to 48%, suggesting that there was significant risk of type II errors. These effect sizes are lower than those previously reported in other cohorts as illustrated in Table 3-8, which summarises the effect sizes from 4,800 cases and 16,289 controls published in 2009 (de Menthon et al., 2009). In this paper, the authors found that the pooled OR for *HLA-B*51*

was 5.90 (95% CI 4.87-7.16). One of the limitations cited by the authors in this meta-analysis was the considerable heterogeneity found in the literature. The effect of including 25% non-Europeans in our cohort had a small but significant effect on the OR. Many cohorts do not fully investigate the ethnicities of participants; thus, any attributable heterogeneity may remain unaccounted for.

Recent data from Ombrello et al. in 2014 found the OR for *HLA-B*51* to be 3.0 (95% CI 2.6-3.4) in a large Turkish cohort of 1190 BD cases and 1257 controls (Ombrello, Kirino, et al., 2014). In 2017, the same group used the Immunochip to identify immunogenetic loci implicated in BD in 1900 BD cases and 1779 controls and found the OR to be 3.26 (95% CI 2.89-3.68) (Takeuchi et al., 2017).

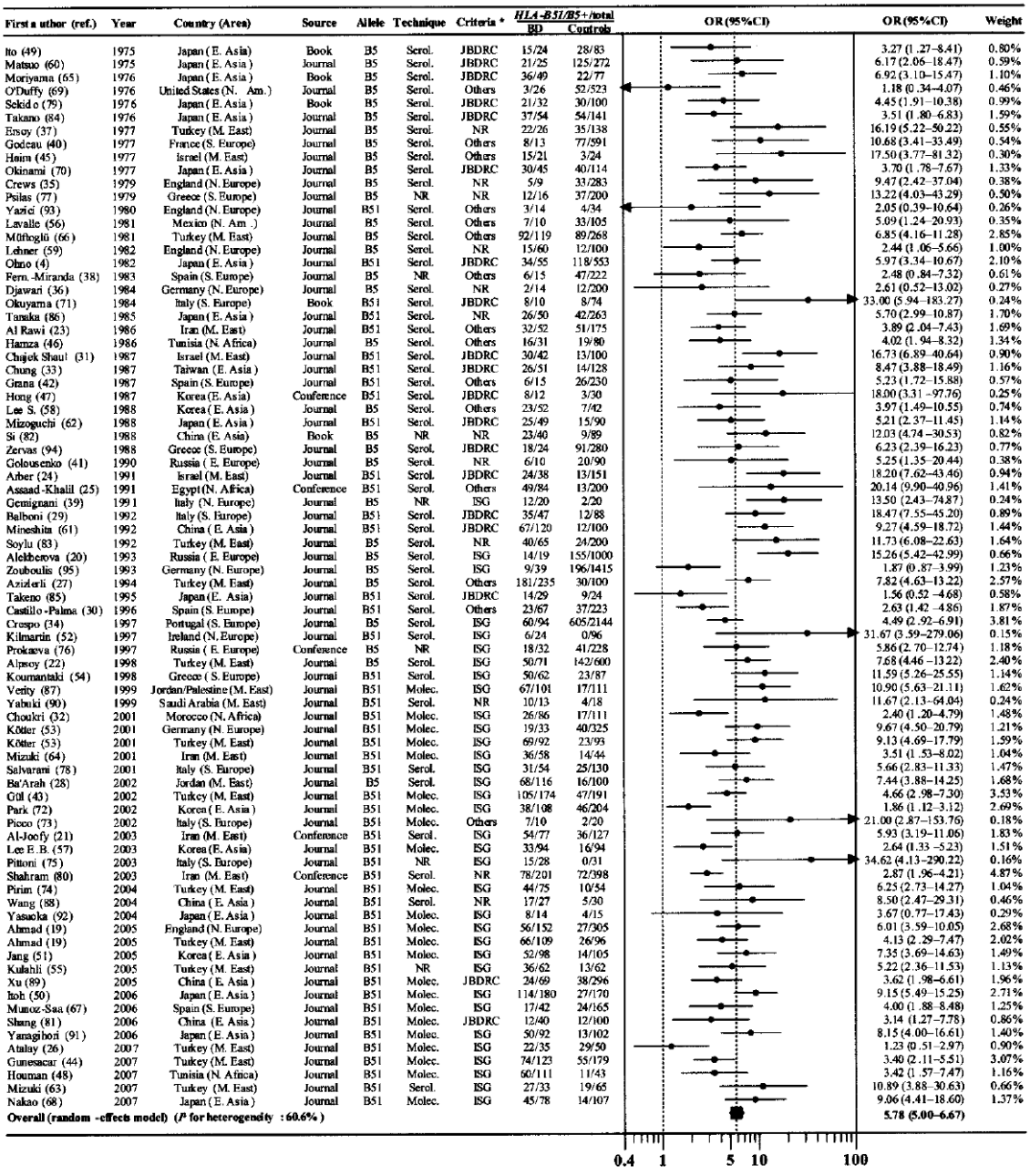
A number of other disease-susceptibility loci have been reported within and around the short arm of chromosome 6. A possible association between *HLA-B*18*, *A*25* and *A*26* and BD was found, all of which were abrogated when controlling for multiple testing. In 2002, Gul et al. found a weak protective effect from *HLA-B*18* in a Turkish cohort (Gul et al., 2002). This was replicated in 2013, in a Spanish population (Montes-Cano et al., 2013). Both of these effects were eliminated when correcting for multiple testing. There are no studies which support the finding that *HLA-B*18* is associated with BD. An explanation for this finding could be the relative paucity of studies that have reported HLA allele frequencies in Eastern Europe, where *HLA-B*18* is most commonly found (Figure 3-9).

HLA-A associations are also recognised in BD. There are no previous reports of an *HLA-A*25* association, but there are several *A*26* associations throughout the literature. Most recently, Al-Okaily et al. reported an association with an OR of 3.25 in a Saudi population

(Al-Okaily et al., 2016). Similar associations have been reported in Taiwanese, Japanese and Greek populations (Y. M. Chung et al., 1990; Kaburaki et al., 2010; Mizuki, Ohno, et al., 1997). Meguro et al. conducted a GWAS in 300 Japanese cases and controls, looking to identify risk loci within and around the MHC (Meguro et al., 2010). The authors reported an OR of 2.07 in the whole cohort and 2.96 in those who were *HLA-B*51*$^{-}$. The majority of the effect was contributed by *HLA-A*26:01*. There was also a significant effect from *HLA-F*01:01:01* and *HLA-G*01:01:02.* There is substantial LD between the implicated *HLA-F* and *–G* loci with *HLA-A*26,* thus *HLA-A*26* was thought to independently increase the risk of BD in this group.

Table 3-8. Forest Plot Of Results Of Published Studies On The Association Between *HLA–B*51/B5* And Behçet's Disease

*Odds ratios for the risk of HLA–B*51/B5 allele carriers to develop BD compared with noncarriers. The dashed vertical line indicates the pooled value for the entire population considered. 95% CI = 95% confidence interval; E = Eastern; Serol. = serologic; JBDRC = Japanese Behçet's Disease Research Committee; N = North; M = Middle; NR = not reported; S = Southern; ISG = International Study Group; Molec. = molecular. Reproduced with permission from (de Menthon et al., 2009).*


First author (ref.) Year Country (Area) Source Allele Technique Criteria * HLA-B51/B5+/total BD Controls OR(95%CI) OR(95%CI) Weight
Ito (49) 1975 Japan (E. Asia) Book B5 Serol. JBDRC 15/24 28/83 3.27 (1.27–8.41) 0.80%
Matsuo (60) 1975 Japan (E. Asia) Journal B5 Serol. JBDRC 21/25 125/272 6.17 (2.06–18.47) 0.59%
Moriyama (65) 1976 Japan (E. Asia) Book B5 Serol. JBDRC 36/49 22/77 6.92 (3.10–15.47) 1.10%
O'Duffy (69) 1976 United States (N. Am.) Journal B5 Serol. Others 3/26 52/523 1.18 (0.34–4.07) 0.46%
Sekido (79) 1976 Japan (E. Asia) Book B5 Serol. JBDRC 21/32 30/100 4.45 (1.91–10.38) 0.99%
Takano (84) 1976 Japan (E. Asia) Journal B5 Serol. JBDRC 37/54 54/141 3.51 (1.80–6.83) 1.59%
Ersoy (37) 1977 Turkey (M. East) Journal B5 Serol. NR 22/26 35/138 16.19 (5.22–50.22) 0.55%
Godeau (40) 1977 France (S. Europe) Journal B5 Serol. Others 8/13 77/591 10.68 (3.41–33.49) 0.54%
Haim (45) 1977 Israel (M. East) Journal B5 Serol. Others 15/21 3/24 17.50 (3.77–81.32) 0.30%
Okinami (70) 1977 Japan (E. Asia) Journal B5 Serol. JBDRC 30/45 40/114 3.70 (1.78–7.67) 1.33%
Crews (35) 1979 England (N. Europe) Journal B5 Serol. NR 5/9 33/283 9.47 (2.42–37.04) 0.38%
Psilas (77) 1979 Greece (S. Europe) Journal B5 NR NR 12/16 37/200 13.22 (4.03–43.29) 0.50%
Yazici (93) 1980 England (N. Europe) Journal B51 Serol. Others 3/14 4/34 2.05 (0.39–10.64) 0.26%
Lavalle (56) 1981 Mexico (N. Am.) Journal B5 Serol. Others 7/10 33/105 5.09 (1.24–20.93) 0.35%
Müftoglü (66) 1981 Turkey (M. East) Journal B5 Serol. Others 92/119 89/268 6.85 (4.16–11.28) 2.85%
Lehner (59) 1982 England (N. Europe) Journal B5 Serol. NR 15/60 12/100 2.44 (1.06–5.66) 1.00%
Ohno (4) 1982 Japan (E. Asia) Journal B51 Serol. JBDRC 34/55 118/553 5.97 (3.34–10.67) 2.10%
Fern.-Miranda (38) 1983 Spain (S. Europe) Journal B5 NR Others 6/15 47/222 2.48 (0.84–7.32) 0.61%
Djawari (36) 1984 Germany (N. Europe) Journal B5 Serol. NR 2/14 12/200 2.61 (0.52–13.02) 0.27%
Okuyama (71) 1984 Italy (S. Europe) Book B51 Serol. JBDRC 8/10 8/74 33.00 (5.94–183.27) 0.24%
Tanaka (86) 1985 Japan (E. Asia) Journal B51 Serol. NR 26/50 42/263 5.70 (2.99–10.87) 1.70%
Al Rawi (23) 1986 Iran (M. East) Journal B51 Serol. Others 32/52 51/175 3.89 (2.04–7.43) 1.69%
Hamza (46) 1986 Tunisia (N. Africa) Journal B5 Serol. Others 16/31 19/80 4.02 (1.94–8.32) 1.34%
Chajek Shaul (31) 1987 Israel (M. East) Journal B51 Serol. JBDRC 30/42 13/100 16.73 (6.89–40.64) 0.90%
Chung (33) 1987 Taiwan (E. Asia) Journal B51 Serol. JBDRC 26/51 14/128 8.47 (3.88–18.49) 1.16%
Grana (42) 1987 Spain (S. Europe) Journal B51 Serol. Others 6/15 26/230 5.23 (1.72–15.88) 0.57%
Hong (47) 1987 Korea (E. Asia) Conference B51 Serol. JBDRC 8/12 3/30 18.00 (3.31–97.76) 0.25%
Lee S. (58) 1988 Korea (E. Asia) Journal B5 Serol. Others 23/52 7/42 3.97 (1.49–10.55) 0.74%
Mizoguchi (62) 1988 Japan (E. Asia) Journal B51 Serol. JBDRC 25/49 15/90 5.21 (2.37–11.45) 1.14%
Si (82) 1988 China (E. Asia) Book B5 NR NR 23/40 9/89 12.03 (4.74–30.53) 0.82%
Zervas (94) 1988 Greece (S. Europe) Journal B5 Serol. JBDRC 18/24 91/280 6.23 (2.39–16.23) 0.77%
Golousenko (41) 1990 Russia (E. Europe) Journal B5 Serol. NR 6/10 20/90 5.25 (1.35–20.44) 0.38%
Arber (24) 1991 Israel (M. East) Journal B51 Serol. JBDRC 24/38 13/151 18.20 (7.62–43.46) 0.94%
Assaad-Khalil (25) 1991 Egypt (N. Africa) Conference B51 Serol. Others 49/84 13/200 20.14 (9.90–40.96) 1.41%
Gemignani (39) 1991 Italy (N. Europe) Journal B5 NR ISG 12/20 2/20 13.50 (2.43–74.87) 0.24%
Balboni (29) 1992 Italy (S. Europe) Journal B51 Serol. JBDRC 35/47 12/88 18.47 (7.55–45.20) 0.89%
Mineshita (61) 1992 China (E. Asia) Journal B51 Serol. JBDRC 67/120 12/100 9.27 (4.59–18.72) 1.44%
Soylu (83) 1992 Turkey (M. East) Journal B5 Serol. NR 40/65 24/200 11.73 (6.08–22.63) 1.64%
Alekberova (20) 1993 Russia (E. Europe) Journal B5 Serol. ISG 14/19 155/1000 15.26 (5.42–42.99) 0.66%
Zouboulis (95) 1993 Germany (N. Europe) Journal B5 Serol. ISG 9/39 196/1415 1.87 (0.87–3.99) 1.23%
Azizlerli (27) 1994 Turkey (M. East) Journal B5 Serol. Others 181/235 30/100 7.82 (4.63–13.22) 2.57%
Takeno (85) 1995 Japan (E. Asia) Journal B51 Serol. JBDRC 14/29 9/24 1.56 (0.52–4.68) 0.58%
Castillo-Palma (30) 1996 Spain (S. Europe) Journal B51 Serol. Others 23/67 37/223 2.63 (1.42–4.86) 1.87%
Crespo (34) 1997 Portugal (S. Europe) Journal B5 Serol. ISG 60/94 605/2144 4.49 (2.92–6.91) 3.81%
Kilmartin (52) 1997 Ireland (N. Europe) Journal B51 Serol. ISG 6/24 0/96 31.67 (3.59–279.06) 0.15%
Prokaeva (76) 1997 Russia (E. Europe) Conference B5 NR ISG 18/32 41/228 5.86 (2.70–12.74) 1.18%
Alpsoy (22) 1998 Turkey (M. East) Journal B5 Serol. ISG 50/71 142/600 7.68 (4.46–13.22) 2.40%
Koumantaki (54) 1998 Greece (S. Europe) Journal B51 Serol. ISG 50/62 23/87 11.59 (5.26–25.55) 1.14%
Verity (87) 1999 Jordan/Palestine (M. East) Journal B51 Molec. ISG 67/101 17/111 10.90 (5.63–21.11) 1.62%
Yabuki (90) 1999 Saudi Arabia (M. East) Journal B51 Serol. NR 10/13 4/18 11.67 (2.13–64.04) 0.24%
Choukri (32) 2001 Morocco (N. Africa) Journal B51 Molec. ISG 26/86 17/111 2.40 (1.20–4.79) 1.48%
Kötter (53) 2001 Germany (N. Europe) Journal B51 Molec. ISG 19/33 40/325 9.67 (4.50–20.79) 1.21%
Kötter (53) 2001 Turkey (M. East) Journal B51 Molec. ISG 69/92 23/93 9.13 (4.69–17.79) 1.59%
Mizuki (64) 2001 Iran (M. East) Journal B51 Molec. ISG 36/58 14/44 3.51 (1.53–8.02) 1.04%
Salvarani (78) 2001 Italy (S. Europe) Journal B51 Serol. ISG 31/54 25/130 5.66 (2.83–11.33) 1.47%
Ba'Arah (28) 2002 Jordan (M. East) Journal B5 Serol. ISG 68/116 16/100 7.44 (3.88–14.25) 1.68%
Gül (43) 2002 Turkey (M. East) Journal B51 Molec. ISG 105/174 47/191 4.66 (2.98–7.30) 3.53%
Park (72) 2002 Korea (E. Asia) Journal B51 Molec. ISG 38/108 46/204 1.86 (1.12–3.12) 2.69%
Picco (73) 2002 Italy (S. Europe) Journal B51 Molec. Others 7/10 2/20 21.00 (2.87–153.76) 0.18%
Al-Joofy (21) 2003 Iran (M. East) Conference B51 Serol. ISG 54/77 36/127 5.93 (3.19–11.06) 1.83%
Lee E.B. (57) 2003 Korea (E. Asia) Journal B51 Molec. ISG 33/94 16/94 2.64 (1.33–5.23) 1.51%
Pittoni (75) 2003 Italy (S. Europe) Journal B51 NR ISG 15/28 0/31 34.62 (4.13–290.22) 0.16%
Shahram (80) 2003 Iran (M. East) Conference B51 Serol. NR 78/201 72/398 2.87 (1.96–4.21) 4.87%
Pirim (74) 2004 Turkey (M. East) Journal B51 Molec. ISG 44/75 10/54 6.25 (2.73–14.27) 1.04%
Wang (88) 2004 China (E. Asia) Journal B51 Serol. NR 17/27 5/30 8.50 (2.47–29.31) 0.46%
Yasuoka (92) 2004 Japan (E. Asia) Journal B51 Molec. ISG 8/14 4/15 3.67 (0.77–17.43) 0.29%
Ahmad (19) 2005 England (N. Europe) Journal B51 Molec. ISG 56/152 27/305 6.01 (3.59–10.05) 2.68%
Ahmad (19) 2005 Turkey (M. East) Journal B51 Molec. ISG 66/109 26/96 4.13 (2.29–7.47) 2.02%
Jang (51) 2005 Korea (E. Asia) Journal B51 Molec. ISG 52/98 14/105 7.35 (3.69–14.63) 1.49%
Kulahli (55) 2005 Turkey (M. East) Journal B51 NR ISG 36/62 13/62 5.22 (2.36–11.53) 1.13%
Xu (89) 2005 China (E. Asia) Journal B51 Molec. JBDRC 24/69 38/296 3.62 (1.98–6.61) 1.96%
Itoh (50) 2006 Japan (E. Asia) Journal B51 Molec. ISG 114/180 27/170 9.15 (5.49–15.25) 2.71%
Munoz-Saa (67) 2006 Spain (S. Europe) Journal B51 Molec. ISG 17/42 24/165 4.00 (1.88–8.48) 1.25%
Shang (81) 2006 China (E. Asia) Journal B51 Molec. JBDRC 12/40 12/100 3.14 (1.27–7.78) 0.86%
Yanagihori (91) 2006 Japan (E. Asia) Journal B51 Molec. ISG 50/92 13/102 8.15 (4.00–16.61) 1.40%
Atalay (26) 2007 Turkey (M. East) Journal B51 Molec. ISG 22/35 29/50 1.23 (0.51–2.97) 0.90%
Gunesacar (44) 2007 Turkey (M. East) Journal B51 Molec. ISG 74/123 55/179 3.40 (2.11–5.51) 3.07%
Houman (48) 2007 Tunisia (N. Africa) Journal B51 Molec. ISG 60/111 11/43 3.42 (1.57–7.47) 1.16%
Mizuki (63) 2007 Turkey (M. East) Journal B51 Serol. ISG 27/33 19/65 10.89 (3.88–30.63) 0.66%
Nakao (68) 2007 Japan (E. Asia) Journal B51 Molec. ISG 45/78 14/107 9.06 (4.41–18.60) 1.37%
Overall (random -effects model) (I² for heterogeneity : 60.6%) 5.78 (5.00–6.67)
0.4 1 5 10 100

*HLA-B*58* was present in 2.2% of controls and 0.19% of cases in our cohort and was found significant in its protective effects. The data has post-hoc power of 60% and is therefore at risk of a type II error. The protective effects of *HLA-B*58* have previously been reported in a Spanish cohort with a similar allele frequency. Less robust effects have been reported in Moroccan, Turkish and Italian Cohorts (Choukri et al., 2001; Gul et al., 2002; Kera et al., 1999). It is not appropriate to draw direct comparisons between the effect of *HLA-B*51* and *B*58* in BD for several reasons. Firstly, the risk-effect of *HLA-B*51* in BD is established and has been widely and consistently reported across the literature, whereas there are only a handful of reports of the protective effects of *HLA-B*58*. Secondly, *HLA-B*51* is more frequently found across most populations than *B*58* (Figure 3-9). However, it is notable that both alleles encode the Bw4 epitope that interacts with *KIR3DL1*.

It has been suggested that polymorphisms outside the Bw4 encoding region may have an effect on Bw4-KIR3DL1 affinity (Sanjanwala et al., 2008). Despite *B*51* and *B*58* both encoding the NIALR Bw4 motif, they differ at positions 67 of the α1 domain (Phenylalanine –Methionine); 94,95 and 97 of the α2 domain (threonine, tryptophan, threonine – isoleucine, isoleucine, arginine); 114 and 116 of the α2 domain (asparagine, tyrosine – aspartic acid, serine) and 171 of the α2 domain (histidine – tyrosine). Sanjanwala et al. conducted mutagenesis experiments on *HLA-B*51:01* and *B*15:13* and found that in addition to the substitutions above, *B*15:13* also differed at positions 45, 46 of the α1 domain, 113 of the α2 domain and 194 of the α3 domain. Of these positions, they found that the largest effects of mutagenesis on Bw4 binding were seen when changes were made to positions 67, 116 and 194, with smaller contributions from residues 94, 95, 97, 113, and 171.

Associations between *MICA* alleles and TM repeats in BD were investigated. I found a modest association between *MICA*009* (A6) and BD, with an OR of 1.80. *MICA*019* (A5) was more commonly found in the BD group, but the association did not survive correction for multiple testing. These findings are in keeping with *MICA* data from other populations. Despite the small sample size, a post-hoc power of 75.6% based on an α of 0.05 was calculated, suggesting that the data is slightly underpowered and that there is a chance of type II error.

Despite the *MICA*009* (A6) association, there was no overall A6-effect seen in the cohort (Table 3-6). *MICA*009* is in strong linkage disequilibrium (LD) with *HLA-B*51* (*D'*=0.88 in the UK cohort) and the association found is likely to be due to this LD. The same *MICA* alleles were associated with BD in the European subgroup, but no specific association with a TM repeat.

In the *HLA-B*51*$^{-}$ individuals, there was a *MICA*009* (A6) effect, independent of other *HLA-B* loci. Park et al. found that the TM repeat MICA A6 maintained an effect in the *HLA-B*51*$^{-}$ individuals in a Korean cohort, but did not analyse the alleles that the TM repeats were coded within (Park et al., 2002). However, I was unable to replicate these findings. In none of the analyses run did I find an A6 association.

In this study, *MICA*004* was the most commonly found MICA A6 allele followed by *MICA*009*, *MICA*011* and *MICA*006*. This finding is in accordance with global *MICA* frequencies, where *MICA*004* occurs in up to 27% of Brazilians, 50% of Yoruba Nigerians and 17% of English individuals (Marin et al., 2004; Norris et al., 2001; W. Tian et al., 2003). *MICA*009* is more commonly found across the South and Central Asia (with high frequencies in Brazil and lower frequencies in Europe), *MICA*011* and **006* have

been reported at low levels across the Asia, Europe and South America. All *MICA* allele frequencies were within HWE of those expected.

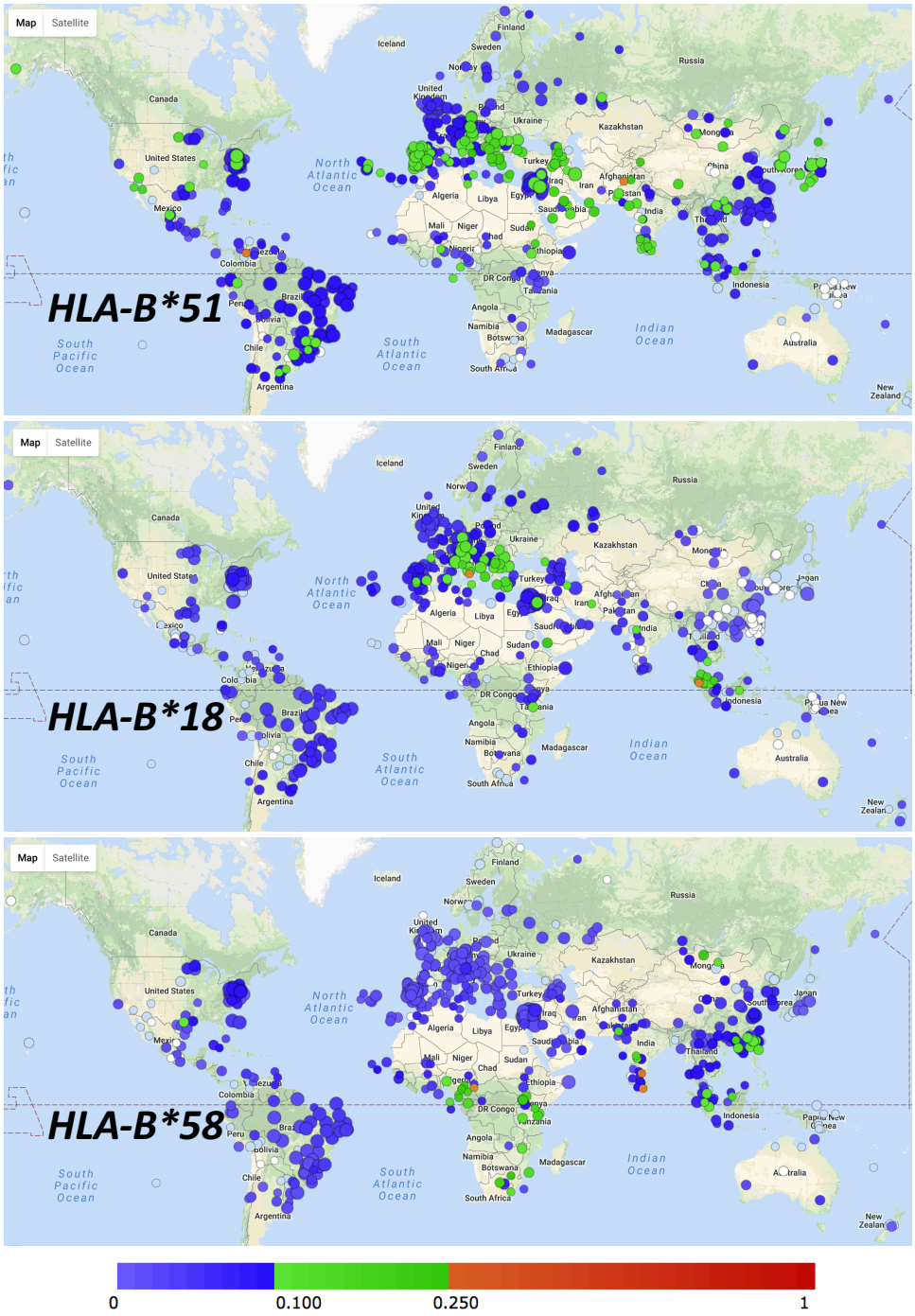
Finally, I examined whether any specific *MICA* associations were found within the ophthalmic group. In the literature, MICA A4 has been associated with ankylosing spondylitis-related uveitis, but not with ankylosing spondylitis (Goto et al., 1997). I was able to identify MICA*009 (A6) as a risk factor for developing BD related uveitis, but did not find any TM repeats associated with eye disease.

3.15 Chapter Summary

In this study, *HLA-B*51* was confirmed as the major risk factor for developing BD in a UK cohort. Furthermore, I identified that the effect of *HLA-B*51* remains a risk factor in a European subgroup, albeit to a lesser degree. *HLA-B*58* was found to be protective in a small number of individuals and *HLA-B*18* a risk allele in *HLA-B*51*$^{-}$ individuals. *HLA-B*51* contributed more risk in those who developed ocular disease compared to MC disease alone. Finally, the association between *MICA*009* (A6) and BD was confirmed in the UK cohort and in those of European ancestry. These findings are important for future work in this patient group. The role of *HLA-B*51* in disease pathogenesis remains elusive. It is possible that the slow assembly of HLA-B*51 relative to other HLA-B allotypes, coupled with its ability to bind peptides promiscuously may result in suboptimal recognition from CD8 T cells. Equally, recent evidence to suggest that ERAP1 variants contribute towards development of BD are intriguing (Guasp et al., 2017).

Figure 3-9. Global Allele Frequencies of *HLA-B*51*, *B*18* and *B*58*.

Blue dots indicate an allele frequency of 0-10%, green 10-25% and orange >25% (www.allelefrequencies.net).



These variants may contribute toward altered peptide trimming leading to altered CD8 T cell recognition, alternatively, the pathogenic role of ERAP1 in BD might be related to its modulation of NK recognition of HLA-B*51. There are previous studies demonstrating that downregulation of ERAP1 expression in human tumour cell lines result NK cell-mediated killing, particularly if target cells express HLA allotypes that bind specific peptides. It might be that however, that the effect of HLA-B*51 in BD is partly explained by its interaction with KIR3DL1 via Bw4. This interaction remains largely unexplored in BD and will be discussed further in the next chapter.

4 *KIR3DL1/S1* Associations And Function In Behçet's Disease

In Chapter 3, I confirmed the genetic associations previously reported in the UK Cohort and demonstrated that conditioning on a European subgroup made little difference to the overall associations. In this chapter, I discuss the impact of *KIR3DL1*/S1 allelic variation in the development of BD on a genetic level. I also attempt to construct a functional system to test the effects of KIR3DL1/S1 allotypes by transfecting various HLA-B constructs into two cell lines from different species.

4.1 *KIR3DL1/S1* Presence/Absence In Behçet's Disease

To investigate allelic variation within *KIR3DL1/S1*, I included amplicons to allow sequence level amplification and analysis of the *KIR3DL1/S1* locus in the NGS panel discussed in Chapter 3 (Appendix 4 – KIR3DL1 Sequencing Primers). After quality control, 256 (95.9%) *KIR3DL1/S1* allotypes were obtained from cases and 433 (92.7%) from controls. I then applied European AIMs to the dataset to derive a European Subgroup. The European subgroup comprised 335 (77% of total) HCs and 198 (76% of total) individuals with BD

There was no difference between the BD and HC population with regard to *KIR3DL1/S1* presence/absence at the gene-level in the UK cohort or the European subgroup (Table 4-1). There was no deviation from Hardy-Weinberg equilibrium in either group (P=0.4533).

Table 4-1. Presence/Absence *KIR3DL1/S1* in Behçet's Disease and demographically matched controls.

There was no association between KIR3DL1/S1 presence/absence in the data from the UK cohort (a.) and the European Subgroup (b.). HC – Healthy Control, BD – Behçet's Disease, OR – Odds Ratio, 95% CI – 95% Confidence intervals.

a.

KIR3DL1/S1	**UK Cohort**						
	HC (n)	**HC (%)**	**BD (n)**	**BD (%)**	***P***	**OR**	**95% CI**
3DL1/3DL1	282	65%	150	59%	0.0878	0.9	0.65-1.24
3DL1/3DS1	137	32%	97	38%	0.0967	1.2	0.87-1.66
3DS1/3DS1	14	3%	9	4%	0.8296	1.09	0.46-2.55

b.

KIR3DL1/S1	**European subgroup**						
	HC (n)	**HC (%)**	**BD (n)**	**BD (%)**	***P***	**OR**	**95% CI**
3DL1/3DL1	205	61%	108	55%	0.2005	0.52	0.24-1.13
3DL1/3DS1	120	36%	81	42%	0.1952	1.44	0.78-2.65
3DS1/3DS1	10	3%	6	3%	1.0000	1.68	0.59-4.75

4.1.1 *KIR3DL1/S1* Presence/Absence In Patients With Ophthalmic Manifestations Of Behçet's Disease

In 2016, Erer et al. reported that patients with ocular manifestations of BD had an increased prevalence of *KIR3DS1* compared to other patients, irrespective of *HLA-B*51* status (Erer et al., 2016). I analysed the ophthalmic subset to ascertain whether I could replicate this finding in the UK cohort. There was no increase in the prevalence of *KIR3DS1* in those with ophthalmic disease. This finding was unrelated to *HLA-B*51* status. There was also no significant difference in the proportion of *KIR3DL1/S1* heterozygotes/homozygotes in the ophthalmic group (P=0.0845).

4.2 *KIR3DL1/S1* Allelic Frequencies

KIR3DL1/S1 alleles were categorised based on their known functional effects (Gardiner et al., 2001; Martin et al., 2007; Sanjanwala et al., 2008; Saunders et al., 2016; Tao et al., 2014) to allow easy visualisation with regard to their functional roles. For those allotypes where functional data was not available, I assigned a presumptive inhibitory-potential based on the nearest KIR3DL1/S1 allotype for which data existed (Table 4-3). *KIR3DL1/S1* allelic frequencies were then calculated in the UK group and the European subgroup. *KIR3DL1*005* was more frequently found in the BD than the HC group, however, the effect was not significant after controlling for multiple tests. No specific *KIR3DL1/S1* allele increased the risk of BD (Table 4-2).

Table 4-2. *KIR3DL1/S1* Allele Frequencies >5% In The UK Cohort And European Subgroup

There are no associations that withstood correction for multiple testing. Alleles with a frequency less than 5% were grouped in the 'Alleles <5%' category. Results in bold signify significant (P<0.05) results. Pc – Bonferroni-Dunn correction for multiple testing, UK – United Kingdom, HC – Healthy Control, BD – Behçet's Disease, OR – Odds Ratio, 95% CI – 95% Confidence intervals. Colour key –Table 4-3

	UK Cohort (HC n=433, BD n=256)								European Subgroup (HC n=335, BD n=198)						
Allele	**HC (n)**	**HC (%)**	**BD (n)**	**BD (%)**	***P***	***Pc***	**OR**	**95% CI**	**HC (n)**	**HC (%)**	**BD (n)**	**BD (%)**	***P***	***OR***	**95% CI**
*3DS1*0130101*	187	22	102	20	0.4937	0.8119	1.08	0.83-1.42	160	24	92	23	0.8234	1.03	0.77-1.38
*3DL1*0010101*	134	15	76	15	0.8161	0.8929	1.04	0.77-1.41	125	19	68	17	0.5653	1.09	0.78-1.5
*3DL1*00401*	119	14	66	13	0.6832	0.8566	1.07	0.77-1.47	120	18	54	14	0.0720	1.31	0.93-1.86
*3DL1*0050101*	109	13	87	17	**0.0255**	0.3348	0.74	0.55-1.01	108	16	82	21	0.0683	0.78	0.57-1.07
*3DL1*002*	106	12	69	13	0.5042	0.8119	0.91	0.66-1.26	87	13	52	13	1.0000	0.99	0.68-1.43
*3DL1*0150201*	59	7	28	5	0.3600	0.8119	1.25	0.78-1.98							
*3DL1*008*	50	6	24	5	0.4584	0.8119	1.23	0.75-2.03							
Alleles < 5%	102	12	60	12					70	10	48	12			

Table 4-3. The Functional Effects Of *KIR3DL1/S1* Alleles Coloured By Their Inhibitory Potential

Where no functional data existed, a presumed effect was assigned based on the nearest KIR3DL1 for which functional data was available.

Functional Effect	Presumed Effect
*3DL1*0010101*	
*3DL1*002*	
*3DL1*00401*	
*3DL1*00402*	
*3DL1*0050101*	
*3DL1*0070101*	
*3DL1*008*	
*3DL1*009*	
*3DL1*01501*	
*3DL1*0150201*	
*3DL1*01701*	
*3DL1*01702*	
*3DL1*019*	
*3DL1*020*	
*3DL1*022*	
*3DL1*025*	
*3DL1*03101*	
*3DL1*033*	
*3DL1*035*	
*3DL1*040*	
*3DL1*052*	
*3DL1*053*	
*3DL1*062*	
*3DL1*063*	
*3DL1*069*	
*3DL1*072*	
*3DS1*0130101*	

Strong Inhibition (KIR3DL1^{HIGH})

Weak Inhibition (KIR3DL1^{LOW})

Null (KIR3DL1^{NULL})

Activating (KIR3DS1)

KIR3DL1/S1 alleles were then grouped by their inhibitory potential to test whether an association existed between high-expressing KIR3DL1 (*KIR3DL1^{HIGH}*), low-expressing KIR3DL1 (*KIR3DL1^{LOW}*), KIR3DL1 retained intracellularly (*KIR3DL1^{NULL}*) or the activating allele *KIR3DS1*. No disease association between KIR3DL1/S1 inhibitory-potential and BD was found.

KIR3DL1/S1 allotypes were analysed by calculating a 'functional-genotype' consisting of the cumulative functional effects of the allotypes present in each group. The combination of a low expressing allele (*KIR3DL1*^{LOW}) and the activating allele *KIR3DS1* was associated with BD (*P*=0.0004, *Pc*=0.0040, OR=2.47, 95% CI 1.43-4.25), whereas a high expressing (*KIR3DL1*^{HIGH}) and null allele (*KIR3DL1*^{NULL}) was associated with protection from disease (*P*=0.0035, *Pc*=0.0350, OR=0.53, 95% CI 0.33-0.87) (Table 4-4).

Table 4-4. Functional Effects Of KIR3DL1/S1 Genotype Combinations.

A risk effect was identified in the BD group for individuals possessing the KIR3DL1^{LOW}*/KIR3DS1 genotype. A protective effect was found in the HC group for individuals possessing the KIR3DL1*^{HIGH}*/KIR3DL1*^{NULL} *genotype. Results in bold signify significant (P<0.05) results. Pc – Bonferroni-Dunn correction for multiple testing, UK – United Kingdom, HC – Healthy Control, BD – Behçet's Disease, OR – Odds Ratio, 95% CI – 95% Confidence intervals. Colour key –Table 4-3*

Functional Genotype		UK Cohort (HC n=433, BD n=256)								European Subgroup (HC n=335, BD n=198)						
Allele 1	*Allele 2*	HC (n)	HC (%)	BD (n)	BD (%)	*P*	*Pc*	OR	95% CI	HC (n)	HC (%)	BD (n)	BD (%)	*P*	OR	95% CI
KIR3DL1^{HIGH}	*KIR3DL1*^{HIGH}	85	17	60	21	0.2467	0.8159	1.20	0.81-1.77	64	18	42	21	0.5755	1.22	0.77-1.94
KIR3DL1^{HIGH}	*KIR3DL1*^{LOW}	71	15	36	14	0.4473	0.9199	0.97	0.62-1.5	47	11	30	15	0.7989	1.17	0.71-1.9
KIR3DL1^{HIGH}	*KIR3DL1*^{NULL}	76	17	24	9	**0.0035**	**0.0315**	0.54	0.33-0.88	61	18	21	9	**0.0186**	0.37	0.2-0.71
KIR3DL1^{HIGH}	*KIR3DS1*	83	19	39	14	0.2155	0.5705	0.77	0.51-1.18	62	16	24	12	0.0671	0.81	0.5-1.31
KIR3DL1^{LOW}	*KIR3DL1*^{LOW}	17	4	7	3	0.5208	0.9077	0.74	0.3-1.82	18	6	5	1	0.1290	1.02	0.37-2.87
KIR3DL1^{LOW}	*KIR3DL1*^{NULL}	23	5	16	6	0.6122	0.9077	1.23	0.63-2.39	18	5	8	4	0.5398	1.47	0.65-3.35
KIR3DL1^{LOW}	*KIR3DS1*	24	6	35	14	**0.0004**	**0.0053**	2.47	1.43-4.25	32	6	33	18	**0.0195**	2.21	1.17-4.19
KIR3DL1^{NULL}	*KIR3DL1*^{NULL}	10	2	7	3	0.8010	0.9077	1.18	0.45-3.15	10	3	9	2	0.3456	0.63	0.17-2.42
KIR3DL1^{NULL}	*KIR3DS1*	30	7	23	9	0.3750	0.8206	1.30	0.74-2.29	21	6	21	11	0.0951	1.53	0.80-2.9
KIR3DS1	*KIR3DS1*	14	3	9	4	0.8296	0.9077	1.09	0.46-2.55	2	3	5	2	0.1078	1.02	0.37-2.87

When the European subgroup was analysed, a similar pattern emerged. The protection conferred from the *KIR3DL1*^{HIGH}*/KIR3DL1*^{NULL} genotype was more robust than the UK cohort as a whole, (*P*=0.0011, OR=0.36, 95% CI 0.19-0.68). Whereas, the risk-effect from the *KIR3DL1*^{LOW}*/KIR3DS1*genotype was lower (*P*=0.0109, OR=2.35, 95% CI 1.23-4.47).

To ensure that the presumptive allotypes assigned did not skew the outcome, the analysis was re-run without these allotypes. This did not affect the overall results and the *KIR3DL1*^{HIGH}*/KIR3DL1*^{NULL} and *KIR3DL1*^{LOW}*/KIR3DS1*genotype remained significant (Table 4-5).

Table 4-5. KIR3DL1/S1 'Functional Genotypes' Without Presumed Functional Effects In The Whole UK Cohort And In Those Of European Ancestry

Despite removing the effects from presumptive allotype, a risk effect was identified in the BD group for individuals possessing the KIR3DL1LOW/KIR3DS1 genotype. A protective effect was found in the HC group for individuals possessing the KIR3DL1HIGH/KIR3DL1NULL genotype. Alleles in bold signify significant (P<0.05) results. Pc – Bonferroni-Dunn correction for multiple testing, UK – United Kingdom, HC – Healthy Control, BD – Behçet's Disease, OR – Odds Ratio, 95% CI – 95% Confidence intervals. Where n=0, OR was calculated using the Haldane correction. Colour key – Table 4-3

Functional Genotype		UK Cohort (HC n=433, BD n=256)								European Subgroup (HC n=335, BD n=198)						
Allele 1	***Allele 2***	**HC (n)**	**HC (%)**	**BD (n)**	**BD (%)**	***P***	***Pc***	**OR**	**95% CI**	**HC (n)**	**HC (%)**	**BD (n)**	**BD (%)**	***P***	**OR**	**95% CI**
KIR3DL1^{HIGH}	*KIR3DL1*^{HIGH}	75	17	53	21	0.3107	0.8159	1.20	0.81-1.77	60	18	42	21	0.3634	1.22	0.77-1.94
KIR3DL1^{HIGH}	*KIR3DL1*^{LOW}	63	15	36	14	0.9108	0.9199	0.97	0.62-1.5	40	12	29	15	0.4232	1.17	0.71-1.9
KIR3DL1^{HIGH}	*KIR3DL1*^{NULL}	75	17	24	9	**0.0048**	**0.0315**	0.54	0.33-0.88	59	18	21	11	**0.0326**	0.37	0.2-0.71
KIR3DL1^{HIGH}	*KIR3DS1*	81	19	37	14	0.1738	0.5705	0.77	0.51-1.18	54	16	21	11	0.0934	0.81	0.5-1.31
KIR3DL1^{LOW}	*KIR3DL1*^{LOW}	16	4	7	3	0.6615	0.9077	0.74	0.3-1.82	18	5	5	3	0.1290	1.02	0.37-2.87
KIR3DL1^{LOW}	*KIR3DL1*^{NULL}	22	5	16	6	0.6048	0.9077	1.23	0.63-2.39	14	4	6	3	0.6391	1.47	0.65-3.35
KIR3DL1^{LOW}	*KIR3DS1*	24	6	35	14	**0.0004**	**0.0053**	2.47	1.43-4.25	26	8	33	17	**0.0024**	2.21	1.17-4.19
KIR3DL1^{NULL}	*KIR3DL1*^{NULL}	10	2	7	3	0.8010	0.9077	1.18	0.45-3.15	9	3	8	4	0.4472	0.63	0.17-2.42
KIR3DL1^{NULL}	*KIR3DS1*	30	7	23	9	0.3750	0.8206	1.30	0.74-2.29	19	6	18	9	0.1583	1.53	0.80-2.9
KIR3DS1	*KIR3DS1*	14	3	9	4	0.8296	0.9077	1.09	0.46-2.55	11	3	4	2	0.5889	1.02	0.37-2.87
KIR3DL1^{HIGH}	***Unknown***	13	3	6	2	0.8104	0.9077	0.78	0.29-2.08	16	5	6	3	0.3757	0.70	0.24-2.03
KIR3DL1^{LOW}	***Unknown***	5	1	0	0	0.1634	0.5705	0.31	0.04-2.60	5	1	1	1	0.4197	0.38	0.04-3.3
KIR3DL1^{NULL}	***Unknown***	1	0	0	0	1.0000	0.9379	1.13	0.09-14.20	2	1	2	1	0.6302	1.14	0.09-14.37
KIR3DS1	***Unknown***	4	1	3	1	0.7148	0.9077	0.85	0.08-9.37	2	1	2	1	0.6302	0.85	0.08-9.46

I then analysed the allelic composition of the implicated 'functional genotypes'. In the UK cohort, there were two genotypes associated with BD: *KIR3DL1*0050101*^{LOW}*/KIR3DS1*0130101* (*P*=0.0210, OR 1.91 95% CI 1.07-3.43) and *KIR3DL1*0070101*^{LOW}*/KIR3DS1*0130101* (*P*=0.0008, OR 15.22, 95% CI 1.92-120.87). Conversely, *KIR3DL1*0010101*^{HIGH}*/KIR3DL1*00401*^{NULL} (*P*=0.0172, OR 0.36, 95% CI

0.15-0.89) was found to be protective (Table 4-6). When the European subgroup was analysed, only *KIR3DL1*0050101*^{LOW}*/KIR3DS1*0130101* (*P*=0.0012, OR 3.26 95% CI 1.46-7.27) retained significance in the risk genotype and there were no specific alleles implicated in the protective genotype.

In order to examine whether the *KIR3DL1/S1* 'functional-genotypes' conferring risk and protection are associated with BD, or merely the presence of Bw4/*HLA-B*51* the above analysis was repeated, controlling for these variables.

Table 4-6. *KIR3DL1/S1* Genotypes Leading To Increased/Decreased Risk Of Developing Behçet's Disease

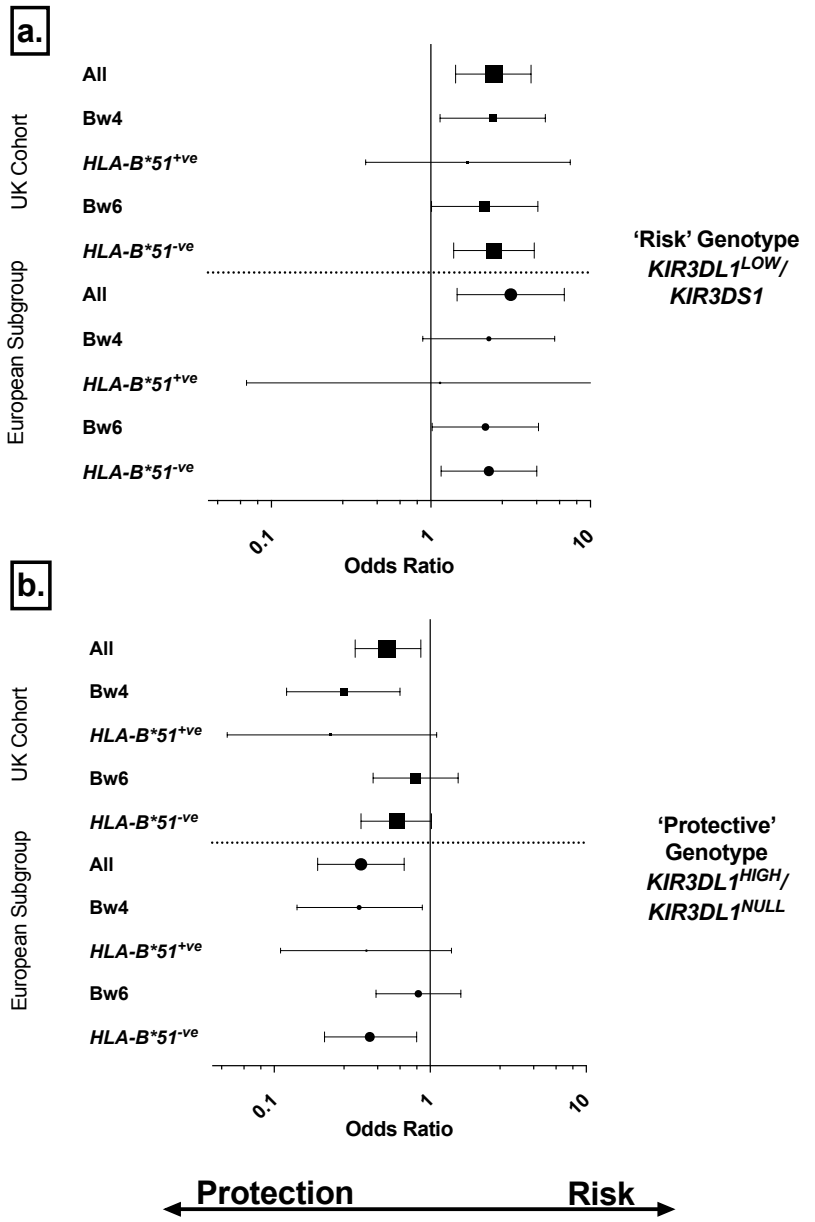
The risk genotype was made up of two KIR3DL1^{LOW} *allotypes – KIR3DL1*005 and KIR3DL1*007. There was only one allotype from the KIR3DL1*^{HIGH} *group – KIR3DL1*001. Those in bold signify significant (P<0.05) results. UK – United Kingdom, HC – Healthy Control, BD – Behçet's Disease, OR – Odds Ratio, 95% CI – 95 % Confidence interval, Colour key – Table 4-3*

KIR3DL1/S1 allele		**UK Cohort (HC n=433, BD n=256)**								**European Subgroup (HC n=335, BD n=198)**						
		HC (n)	**HC (%)**	**BD (n)**	**BD (%)**	***P***	**OR**	**95% CI**		**HC (n)**	**HC (%)**	**BD (n)**	**BD (%)**	***P***	**OR**	**95% CI**
Increased Risk																
3DL1*005	***3DS1*013***	23	5.31	26	10.16	**0.0210**	1.91	1.07-3.43		11	5.26	17	17.17	**0.001**	3.3	1.46-7.27
3DL1*007	***3DS1*013***	1	0.23	9	3.64	**0.0008**	15.2	1.92-120.87		1	0.48	1	1.01	0.063	2.1	0.13-34.11
Decreased Risk																
3DL1*001	***3DL1*004***	6	2.34	28	6.47	**0.0172**	0.36	0.15-0.89		12	5.74	2	2.02	0.059	0.4	0.08-1.60

In the UK cohort, the effect of possessing a *KIR3DL1*^{LOW}*/KIR3DS1* 'risk' genotype was significant in all groups except the *HLA-B*51*$^{+}$ individuals. The protective effect of the *KIR3DL1*^{HIGH}*/KIR3DL1*^{NULL} 'protective' genotype was only significant in individuals possessing Bw4 (Figure 4-1). When the data was conditioned on the European subgroup, the effect of possessing Bw4 was abrogated in the *KIR3DL1*^{LOW}*/KIR3DS1* 'risk' genotype, whereas it was maintained in the *KIR3DL1*^{HIGH}*/KIR3DL1*^{NULL} genotype.

Figure 4-1. The Effect Of Associated *KIR3DL1/S1* 'Functional Genotypes' By Subgroup

*In the UK cohort and European subgroup, significant risk effects were found in the Bw4 and HLA-B*51- groups. Significant protective effects in the UK cohort, were found in the Bw4 group only, whereas the protective effects were found in both the Bw4 and HLA-B*51- groups in the European subgroup. Odds Ratios & 95% CIs for the KIR3DL1^{LOW}/KIR3DS1 'risk' (a.) and KIR3DL1^{HIGH}/KIR3DL1^{NULL} 'protective' genotypes (b.) for the UK cohort and the European subgroup. The size of the marker is proportional to the size of the group. All – All individuals possessing genotype; Bw4 – individuals possessing at least one allele encoding the Bw4 epitope; Bw6 – individuals who lack an allele encoding Bw4; HLA-B*51+ – individuals who possess at least allele encoding HLA-B*51; HLA-B*51- – individuals who lack an allele encoding HLA-B*51.*



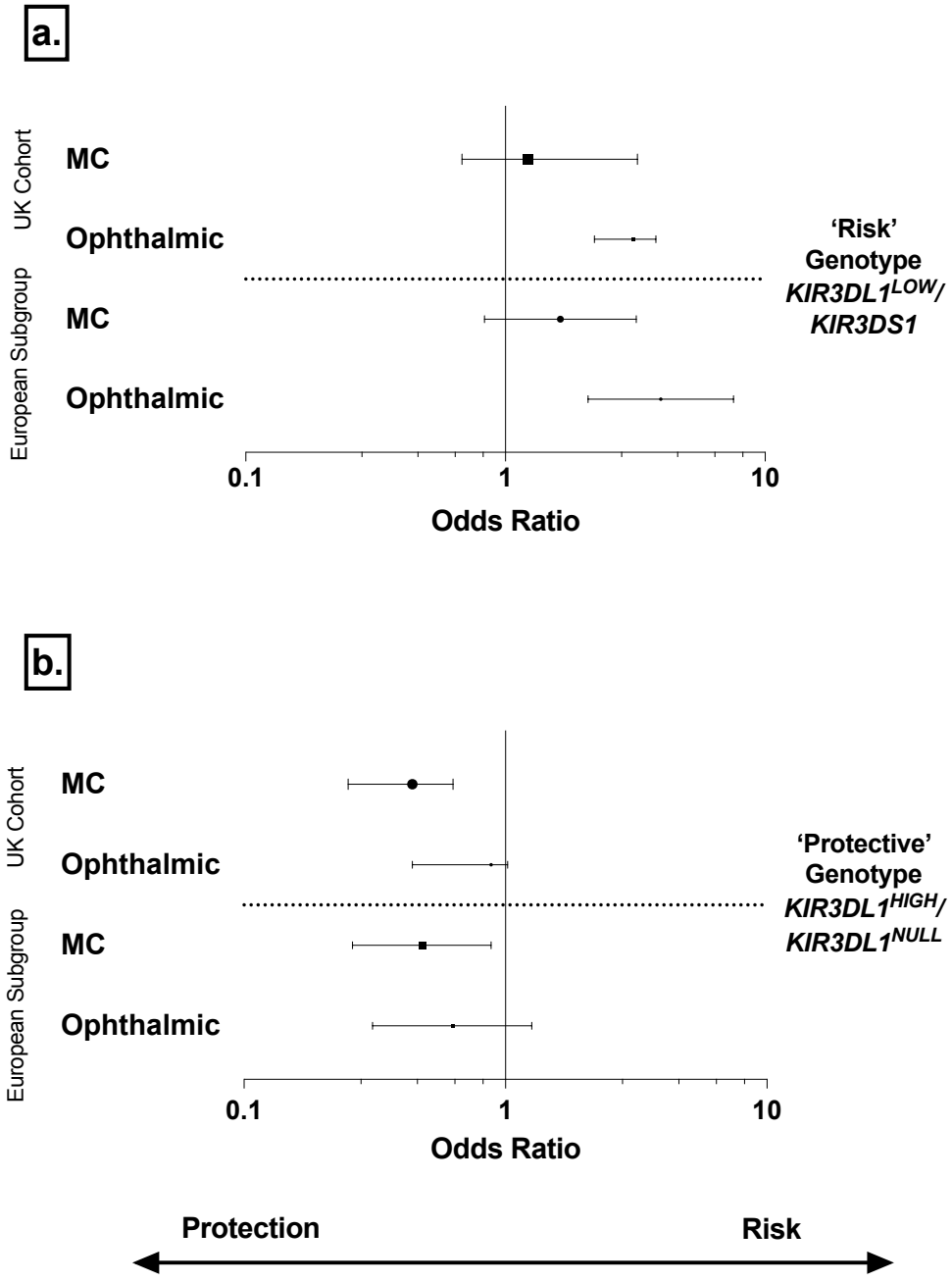
As discussed in Chapter 3, the BD population was then split into two subgroups; those with purely mucocutaneous (MC) disease (n=166) and those with mucocutaneous disease and ophthalmic involvement (n=80). No significant differences were found between the two groups with regard to *KIR3DL1/S1* presence/absence and were unable to replicate the association between an increase in *KIR3DS1* alleles and ophthalmic involvement as has been previously reported (Erer et al., 2016).

The cumulative 'functional-genotypes' did not show any association between $KIR3DL1^{LOW}/KIR3DS1$ and MC disease, However, examining the allele frequencies in the MC group revealed an increase in $KIR3DL1*00701^{LOW}/KIR3DS1*013$ compared to HCs (P=0.0071, OR 13.04, 95% CI 1.51-112.49). Conversely, the $KIR3DL1^{HIGH}/KIR3DL1^{NULL}$ genotype did reduce the risk of developing MC disease (P=0.0048, OR 0.45, 95% CI 0.25-0.81), but no specific allelic combination was significant.

The ophthalmic group had significant risk conferred from the $KIR3DL1^{LOW}/KIR3DS1$ genotype (P=5.76x10^{-5}, OR 3.92, 95% CI 2.06-7.47), (Figure 4-2). Both $KIR3DL1*00501^{LOW}/KIR3DS1*013$ (P=0.0003, OR 3.27, 95% CI 1.65-6.48) and $KIR3DL1*00701^{LOW}/KIR3DS1*013$ (P=0.0039, OR 18.83, 95% CI 2.08-170.47) were significant in making up the 'functional genotype'. There were no protective effects observed in this group.

Figure 4-2. *KIR3DL1/S1* 'Functional Genotypes' In Mucocutaneous And Ophthalmic Disease

In the UK cohort and European subgroup, significant risk effects were found in the ophthalmic groups only, whereas significant protective effects were only found in the MC groups. KIR3DL1^{LOW}/KIR3DS1 'risk' (a.) and KIR3DL1^{HIGH}/KIR3DL1^{NULL} 'protective' genotypes (b.) for the UK cohort and the European subgroups. The size of the marker is proportional to the size of the group. MC – Mucocutaneous disease only; Ophthalmic – Behçet's Disease with ocular manifestations.



4.3 Functional Assays To Examine The Expression Of KIR3DL1/S1 On Circulating Lymphocytes In Behçet's Disease

The aim of the functional work in this Chapter was to examine the phenotype of NK cells and KIR3DL/S1 expression in patients with BD. I then hoped to carry out cytotoxicity assays using the cell lines expressing HLA-B*51 (the primary HLA allele implicated in BD). HLA-B*52 (an allele very similar to HLA-B*51, but not implicated in BD) and finally HLA-B*35 (an allele that expresses Bw6, rather than Bw4 and therefore is not a ligand for KIR3DL1, nor is it associated with BD). I created a number of cell lines to test patient's PBMCs against. First, I created adherent CHO-cell lines that do not express any human ligands for HLA or KIR. Second, I created non-adherent 221 cell lines, which express activating receptors and B2M, but not HLA Class-I.

At the start of the project, I planned to recall patients after *KIR3DL/S1* genotyping had completed, thus giving me the opportunity to test specific combinations of PBMC grouping individuals with comparable *KIR3DL1/S1* genotypes to increase the power of the study. Unfortunately, it was not possible to recall the majority of these patients (discussed below) and I extracted PBMC from only 22 BD patients with known KIR3DL1/S1 allotypes. Therefore the power of the functional work is low and I have kept the results descriptive.

In order to establish the effects of KIR3DL1/S1 allotypes *in vivo* I set out to characterise the NK and CD8 T cell subsets in patients with established KIR3DL1/S1 genotypes. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood and frozen in aliquots to allow experimental repeats to be carried out.

4.4 Medication

Medication is discussed here as the immunosuppressive drugs used to treat BD can have profound effects on lymphocyte populations. Behçet's Disease can be managed in a number of ways and treatment is usually tailored to the needs of each individual. Broadly speaking, mild disease can be managed with oral colchicine and topical steroids during a flare (in the form of mouthwash for oral/pharyngeal ulceration or ointment for genital ulceration). Moderate disease is managed in the same way as mild disease, but with the addition of a disease-modifying drug such as azathioprine or mycophenolate mofetil. Severe disease is associated with significant morbidity and mortality. It is treated with high-dose systemic corticosteroids or a biologic agent such as an anti-TNFα monoclonal antibody plus one or more disease modifying drugs. I have not focused on the effects of medication in BD in this thesis, however it is important to be aware of the impact immunosuppressive medication may have on the lymphocyte compartment for functional analyses. There is substantial evidence that antiproliferative DMARDs such as azathioprine reduce circulating NK cells and γδT cells (Cseuz et al., 1990; Czeuz et al., 1990; McCarthy et al., 2015; Orandi et al., 2017; Pedersen et al., 1986; Vacher-Coponat et al., 2006). Figure 4-4b shows the breakdown of patients taking biologic agents and disease-modifying drugs. Seven (31.9%) of the 22 donors were taking an anti-proliferative agent at the time of venepuncture.

4.5 Natural Killer Cell Phenotypes In Behçet's Disease Patients With Known KIR3DL1/S1 Allotypes.

4.5.1 Circulating Natural Killer Cells Are Present In Low Numbers In Behçet's Disease

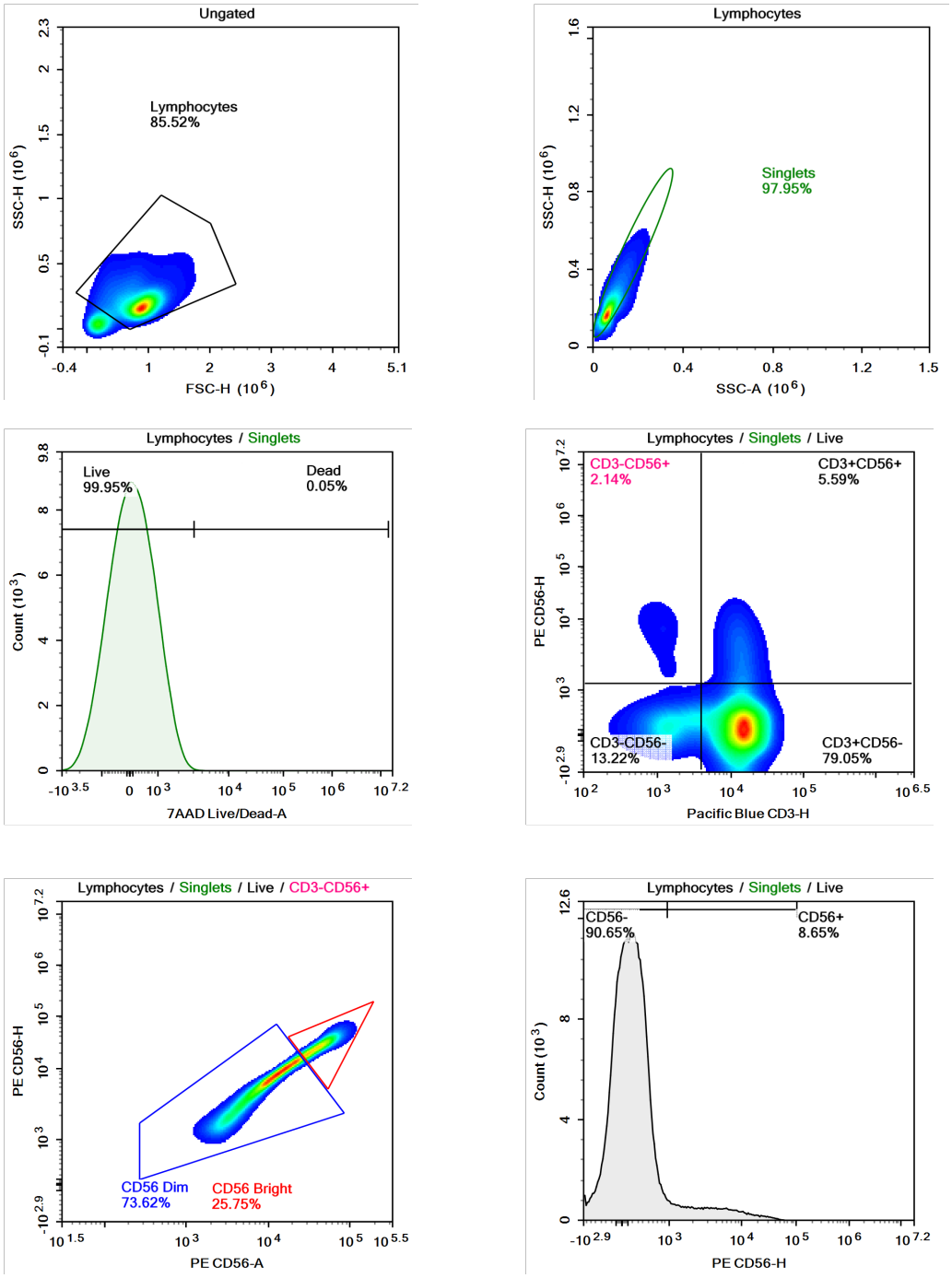
NK cells were identified as $CD3^{-}CD56^{+}$. Results are expressed as a percentage of single, live-gated lymphocytes in peripheral blood (Figure 4-3). BD patients had low percentages of circulating NK cells - 5.35 ± 2.31% (Figure 4-4), with $CD3^{-}CD56^{Bright}$ NK cells making up 9.86 ± 3.82% of the NK cell compartment. There was no correlation between KIR3DL1/S1 allotype and % NK cells (r^2 = -0.299, *P*=0.5456), nor was there a correlation between antiproliferative medications and % NK cells (r^2 = 0.0126, *P*=0.7625).

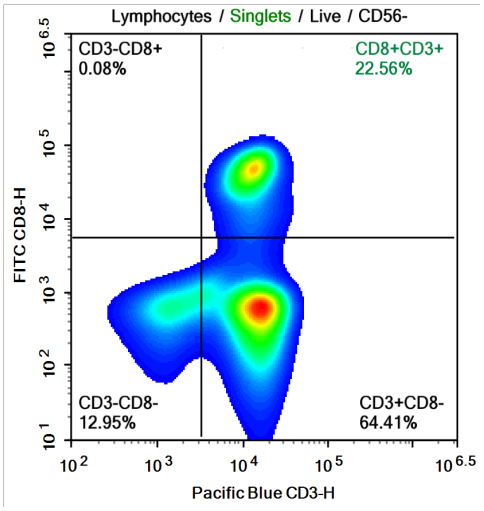
4.5.2 Disease Activity Does Not Affect Natural Killer Cell Percentages

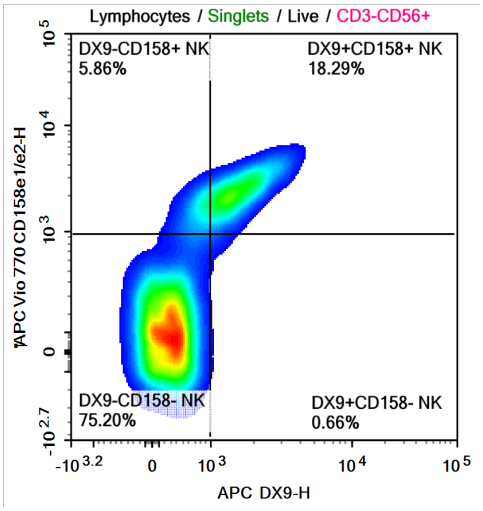
Of the 22 individuals recruited for phenotypic analysis. Twelve (54.5%) individuals were inactive at the time of venepuncture and 10 (45.5%) were active in one or more systems. There was no difference in levels of circulating NK cells between the active and inactive groups (*P*=0.6524).

Figure 4-3. Gating Strategy Used To Identify Natural Killer Cells

$CD56^{Bright}$ NK cells ($CD3^{-}CD56^{Bright}$), $CD56^{Dim}$ NK cells ($CD3^{-}CD56^{Dim}$), $KIR3DL1^{+}$ cells ($DX9^{+}$) and $KIR3DS1^{+}$ cells ($REA\ 618^{+}$). Density plots and histograms from a representative individual (Sample 14) expressing KIR3DL1/S1. In this individual there were 2.14% circulating NK cells. NK - Natural killer cell, Dim - $CD56^{Dim}$, Bright - $CD56^{Bright}$, CD8 T cell - $CD3^{+}CD8^{+}$ T cell.




Lymphocytes / Singlets / Live / CD56-
CD3-CD8+
0.08%
CD8+CD3+
22.56%
CD3-CD8-
12.95%
CD3+CD8-
64.41%
FITC CD8-H
Pacific Blue CD3-H


Lymphocytes / Singlets / Live / CD3-CD56+
DX9-CD158+ NK
5.86%
DX9+CD158+ NK
18.29%
DX9-CD158- NK
75.20%
DX9+CD158- NK
0.66%
APC Vio 770 CD158e1/e2-H
APC DX9-H

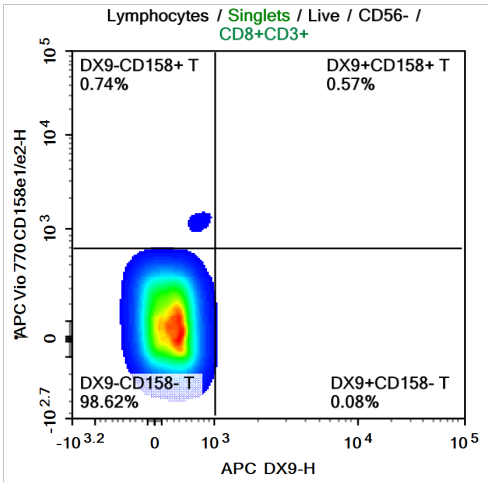
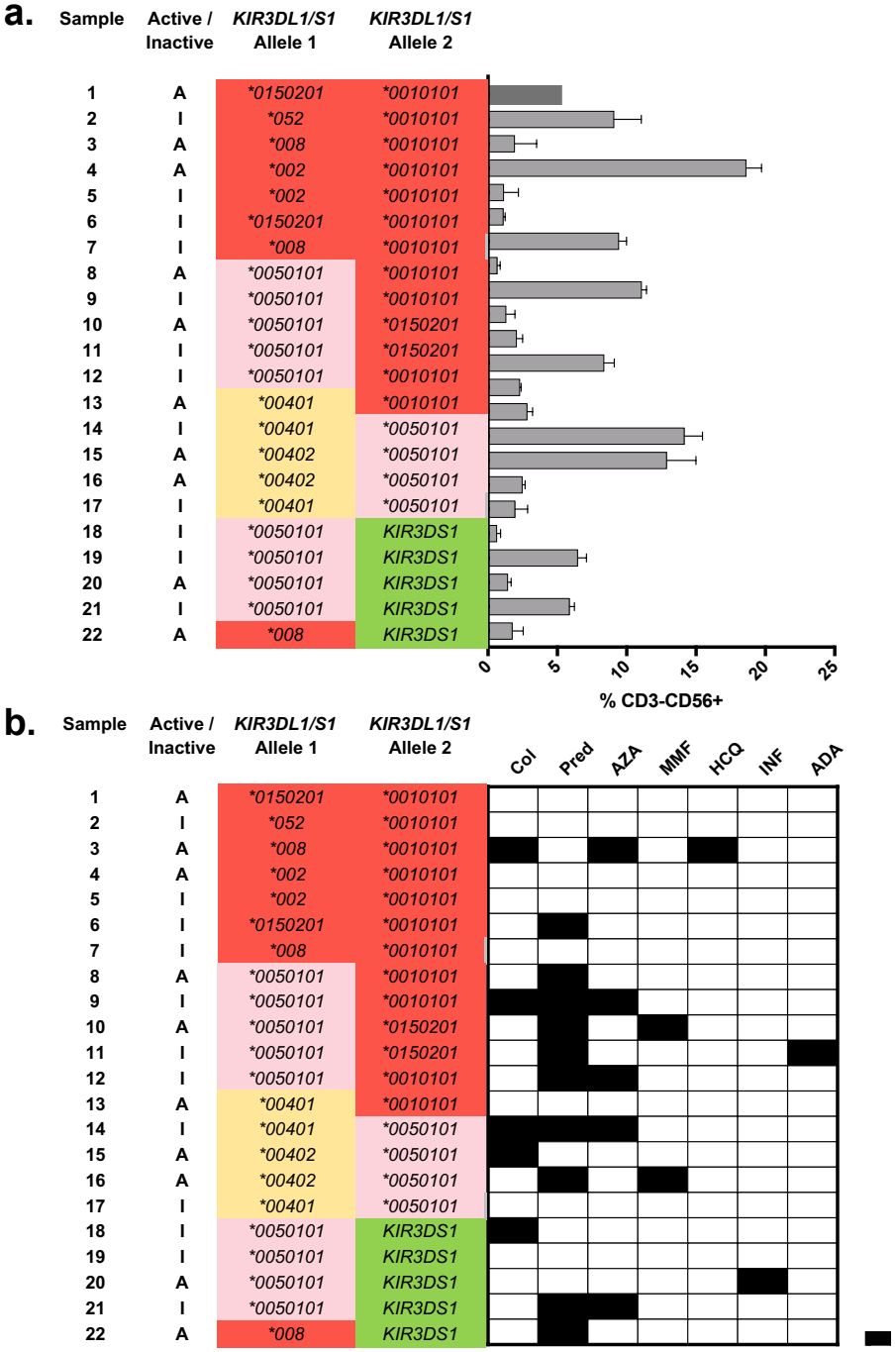

Lymphocytes / Singlets / Live / CD56- /
CD8+CD3+
DX9-CD158+ T
0.74%
DX9+CD158+ T
0.57%
DX9-CD158- T
98.62%
DX9+CD158- T
0.08%
APC Vio 770 CD158e1/e2-H
APC DX9-H

Figure 4-4. (a.) Percentage Of Circulating $CD3^{-}CD56^{+}$ Cells In Individuals With Known KIR3DL1/S1 Allotypes. (b.) Medications Taken By Individuals At The Time Of Venepuncture

(a.) Low percentages of circulating NK cells were found in the BD patients. Measurements were repeated an average of 3 times using frozen aliquots of PBMC. (b.) Subjects' research numbers are shown next to their KIR3DL1/S1 allotypes Medications taken at the time of venepunture. Col – colchicine, Pred – prednisolone, AZA – azathioprine, MMF – mycophenolate mofetil, HCQ – hydroxychloroquine, INF – infliximab, ADA – adalimumab



4.5.3 KIR3DL1/S1 Is Variably Expressed On Circulating Natural Killer Cells

Peripheral blood mononuclear cells were labelled with an anti-KIR3DL1 antibody (DX9 clone – henceforth DX9), which recognises KIR3DL1, but not KIR3DS1 and an anti-KIR3DL1/S1 antibody (REA 618 clone, henceforth REA 618). Using this combination of antibodies, KIR3DS1 populations can be identified.

Levels of DX9 binding are known to vary between individuals, a phenomenon attributed partly to *KIR3DL1* allelic variation and the presence of self-ligands for KIR3DL1 (Burian et al., 2016; Trundley et al., 2007). I found the majority of donors' NK cells bound DX9 and REA 618 weakly. Eleven (50%) individuals expressed <1% of KIR3DL1 on their surface. Four (18%) expressed 1-5% and 7 (32%) expressed >5% KIR3DL1. Overall, KIR3DL1 was expressed in 5.22 ± 3.02% of NK cells. Seven individuals (32%) possessed a KIR3DS1 allotype. Expression of KIR3DS1 was found to be low in the individuals analysed. Five (71%) expressed <1% KIR3DS1 (Figure 4-5).

The presence/absence of Bw4 was analysed to determine whether possession of the ligand for KIR3DL1 increased its expression in circulating NK cells. No differences were identified between those bearing Bw4 and those without it (P=0.0725).

Figure 4-5. Heatmap Showing The Percentage KIR3DL1/S1 Expression On Natural Killer Cells

Surface expression of KIR3DL1 and KIR3DS1 were strongly correlated, however both receptors were expressed at low levels. Each individual's HLA-A and –B type is shown in low-resolution and colour coded grey if the allele encodes Bw4. P<0.05 was considered significant. NK – Natural killer, A – Active, I - Inactive

Sample	Active / Inactive	*KIR3DL1/S1* Allele 1	*KIR3DL1/S1* Allele 2	HLA-A Allele 1	HLA-A Allele 2	HLA-B Allele 1	HLA-B Allele 2	% NK KIR3DL1 Expression	% NK KIR3DS1 Expression
1	A	*0150201	*0010101	A*02	A*11	B*39	B*39	1.66	NA
2	I	*052	*0010101	A*03	A*32	B*07	B*35	5.00	NA
3	A	*008	*0010101	A*01	A*03	B*51	B*52	2.00	NA
4	A	*002	*0010101	A*01	A*03	B*08	B*18	13.00	NA
5	I	*002	*0010101	A*01	A*32	B*08	B*08	22.00	NA
6	I	*0150201	*0010101	A*02	A*03	B*35	B*35	4.00	NA
7	I	*008	*0010101	A*24	A*11	B*51	B*08	17.00	NA
8	A	*0050101	*0010101	A*03	A*29	B*08	B*41	16.00	NA
9	I	*0050101	*0010101	A*01	A*02	B*52	B*53	0.00	NA
10	A	*0050101	*0150201	A*03	A*08	B*35	B*18	0.00	NA
11	I	*0050101	*0150201	A*02	A*11	B*08	B*40	0.00	NA
12	I	*0050101	*0010101	A*02	A*34	B*08	B*14	0.00	NA
13	A	*00401	*0010101	A*03	A*74	B*18	B*42	3.00	NA
14	I	*00401	*0050101	A*01	A*68	B*27	B*44	10.00	NA
15	A	*00402	*0050101	A*02	A*11	B*13	B*51	3.00	NA
16	A	*00402	*0050101	A*02	A*03	B*07	B*15	2.00	NA
17	I	*00401	*0050101	A*24	A*31	B*40	B*51	2.00	NA
18	I	*0050101	KIR3DS1	A*02	A*30	B*08	B*35	19.00	5
19	I	*0050101	KIR3DS1	A*01	A*02	B*56	B*57	2.00	1
20	A	*0050101	KIR3DS1	A*01	A*03	B*40	B*51	3.00	2
21	I	*0050101	KIR3DS1	A*02	A*02	B*40	B*51	4.00	2
22	A	*008	KIR3DS1	A*02	A*68	B*08	B*51	3.00	1

=Bw4

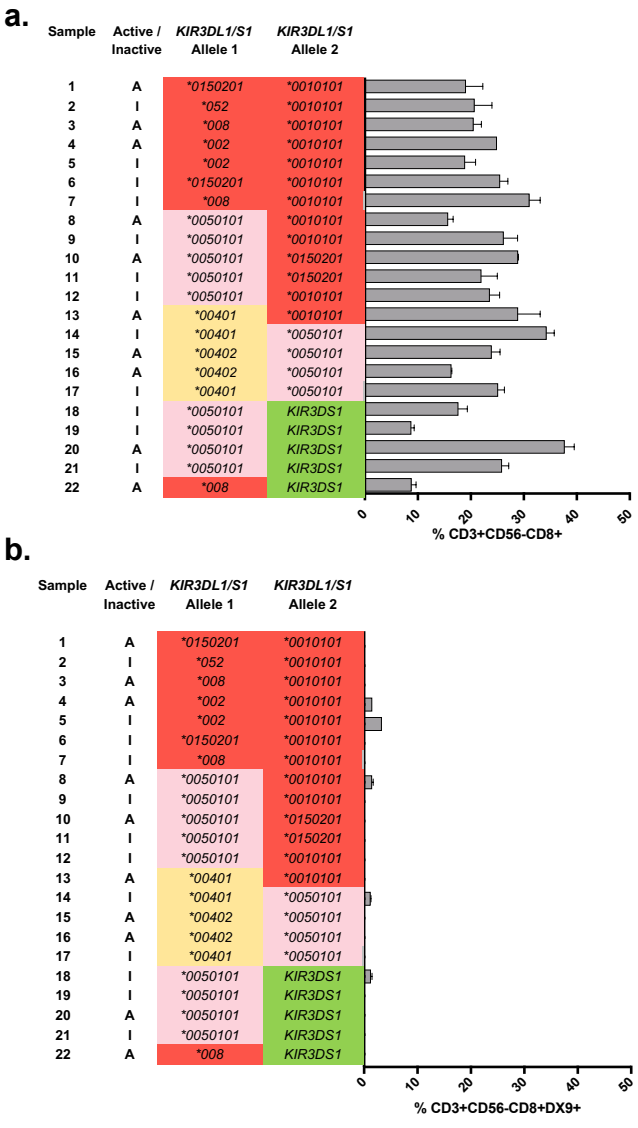
4.5.4 Circulating CD8 T Cells Minimally Express KIR3DL1/S1 On The Cell Surface

CD8 T cells also express KIR. Previous work has suggested that CD8 T cells may express different KIRs to NK cells and that epistatic interaction between HLA ligands and KIR may not affect T cell cytotoxicity in the same way as NK cells (Bjorkstrom et al., 2012). To examine the CD8 T cell compartment, $CD3^{+}CD56^{-}CD8^{+}$ T cells were identified and KIR3DL1/S1 expression analysed. CD8 T cells made up 22.82 ± 3.10% of circulating

lymphocytes in the 22 individuals sampled (Figure 4-6a). KIR3DL1/S1 was measured on circulating T cells using anti-KIR3DL1/S1 antibodies (DX9 and REA 618). No REA 618 and minimal DX9 (0.8 ± 0.71%) bound to circulating CD8 T cells (Figure 4-6b). There was a low-moderate correllation between individuals expressing KIR3DL1 on NK and T cells ($r^2 = 0.49$, P=0.027).

Figure 4-6. Percentage Of Circulating CD8 T Cells In Individuals With Known KIR3DL1/S1 Allotypes And Expression Of KIR3DL1 On CD8 T Cells

(a.) Percentage of circulating CD8 T cells in patients with BD and (b.) DX9$^+$ CD8 T cells. Measurements were repeated an average of 3 times from frozen aliquots of PBMC. Subjects' research numbers are shown next to their KIR3DL1 allotypes as well as their disease state at the time of venepuncture.



4.6 Creation Of A Functional System To Test KIR3DL1/S1 Allotypes

Previous work by Shafi et al (Figure 1-6) indicated that PBMC from individuals with BD had variable degranulation responses when presented with target cells (CHO-cells) expressing HLA-B*51. This phenomenon was not observed when the same PBMC were presented with HLA-B*52^{+} target cells. Possible explanations for this variation include:

1. KIR3DL1/S1 allotypic variation between patient samples. NK cells from an individual with a *KIR3DL1/S1* genotype consistent with strong inhibition (e.g. *KIR3DL1*001/KIR3DL1*01501*) should exhibit less cytotoxic activity on contact with its Bw4 ligand than NK cells with a genotype consistent with weak inhibition (e.g. *KIR3DL1*005/KIR3DL1*007*).
2. NK cells from donors with reduced levels of cytotoxicity may have been previously educated to HLA-B*51 (donor HLA status was not defined in this study).
3. Target cells may have had unstable expression of HLA-B constructs over time (no attempt was made to re-sort target cells over time to maintain an HLA-B -rich population). Furthermore, HLA-B and B2M constructs were cloned into pcDNA3.1(+) plasmids with the same antibiotic resistance gene (neomycin), thus making it impossible to select cells with media enriched with antibiotics.
4. Donors may have been exposed to different medications affecting their lymphocyte function (medication was not reported).

To avoid the above pitfalls, I had the option to remake the CHO target cells either by transfecting the three constructs (MICA*009, HLA-B, B2M) in separate plasmids with three antibiotic resistance genes, or by cloning the three constructs into one larger plasmid with separate promoter sequences to ensure equal expression.

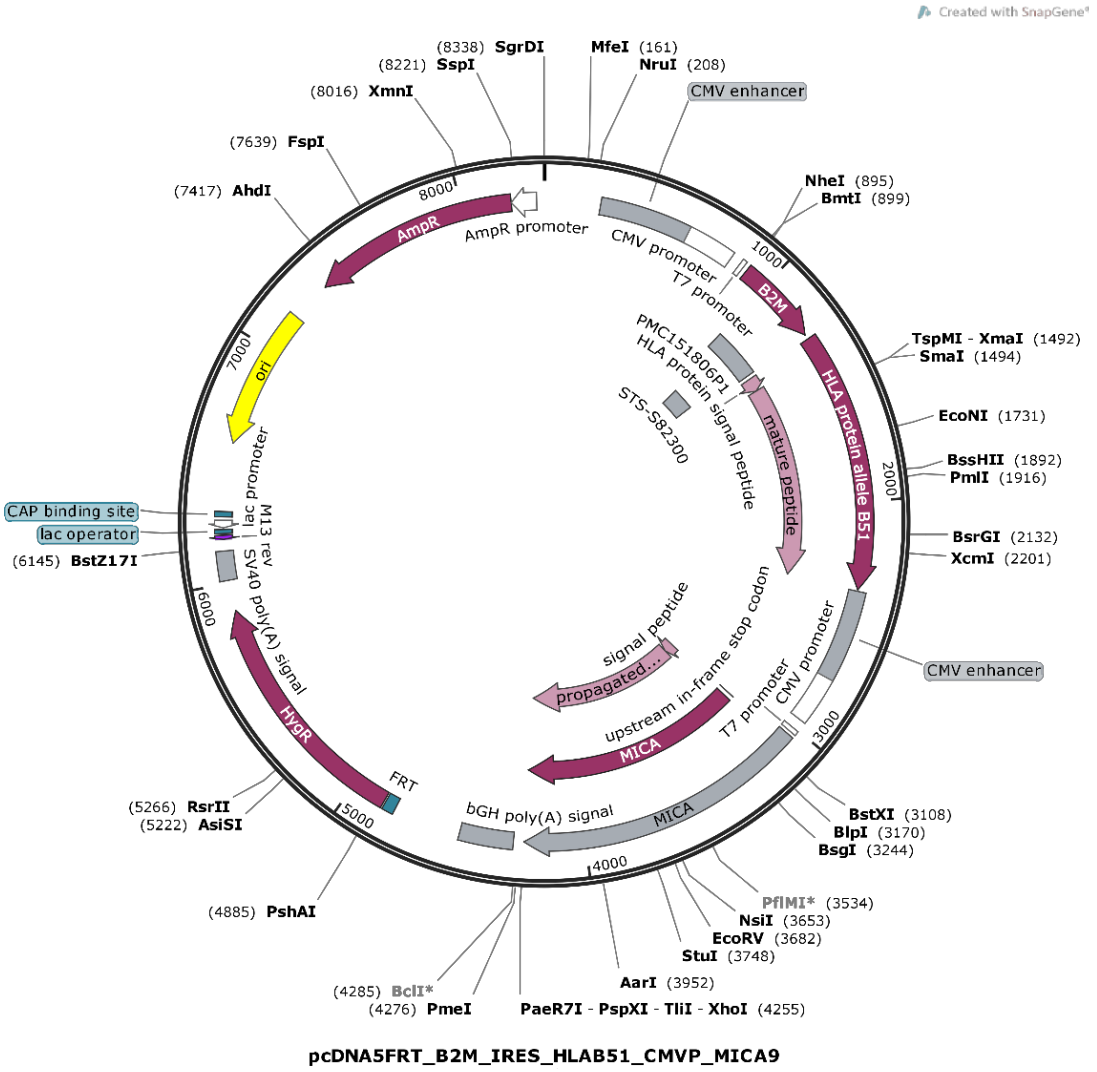
I elected to transfect constructs into one larger pcDNA5-FRT plasmid (Chapter 2.8.1). In addition to HLA-B*51 and B*52, I also chose to transfect HLA-B*35 as a Bw6 control. Instead of cloning the HLA constructs from patients or healthy controls, I synthesised our HLA-B and MICA constructs to ensure accuracy and obtained the shorter and oligomorphic B2M sequence from a healthy control. To reduce the size of the plasmid, I introduced an internal ribosome entry site (IRES) between the B2M and HLA-B constructs. IRES are useful short sequences that allow a ribosome to remain attached to mRNA and start transcribing the next coding sequence without the need for another (larger) promoter region. The three constructs were then assembled using Gibson assembly in a pcDNA5-FRT high copy number plasmid with a hygromycin resistance gene (Figure 4-7).

The plasmids were then amplified in chemically competent NEB 5a *E. Coli* overnight and purified on an agarose gel to ensure the correct size product. The plasmid was digested with restriction enzymes to ensure the correct products were present (Figure 4-8). The insert was then sent for Sanger sequencing to confirm no errors had occurred in the sequence during the cloning procedure.

The pcDNA5-FRT vector contains a hygromycin resistance gene which lacks a start codon, downstream of a homologous FRT site and upstream of the multiple cloning site (MCS), into which our genes of interest (GOI) were inserted. The pcDNA5-FRT vector containing the triple construct was transfected into CHO-FRT cells, which resulted in a homologous recombination event between the FRT sites on the plasmid and in the cell line.

Figure 4-7. pcDNA5-FRT Plasmid With Triple Construct And Hygromycin Resistance Gene.

*Expression of the B2M gene is driven by a CMV promoter. The stop codon of the B2M gene was removed to allow an IRES to be entered immediately downstream. This was followed by the HLA-B gene. Another CMV promoter region was added after this to drive expression of MICA*009. CMV – cytomegalovirus, B2M – Beta 2 Microglobulin, HLA-Human Leukocyte Antigen, MICA – MHC Class I polypeptide-related sequence A. FRT – Flp recombinase target, AmpR – ampicillin resistance gene.*

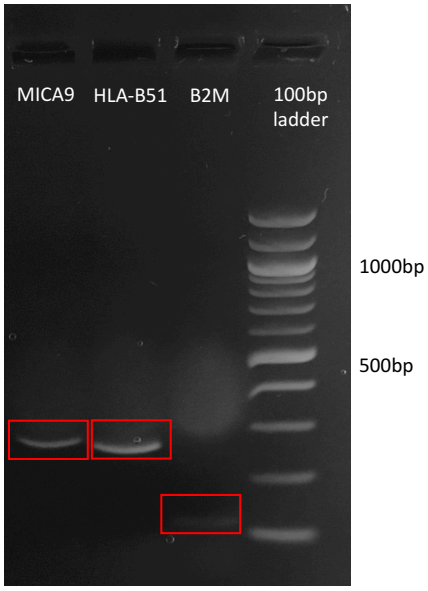


This process has the advantage of integrating the GOI into the CHO-cell genome downstream of a CMV promoter. It also disrupts the zeocin resistance gene present in the CHO-FRT cells and provides a start codon for the hygromycin resistance gene present in the pcDNA5-FRT vector. Positive cells expressing the GOI are then selected for

hygromycin resistance by enriching the media with hygromycin, thus killing all non-transfected cells that retained their functional zeocin resistance gene.

Figure 4-8. Enzyme Digest To Ensure *MICA*009*, *HLA-B*51* And *B2M* Were All Present In The pcDNA5-FRT/*B2M/HLA-B*51*/*MICA*009*/Hygromycin Vector

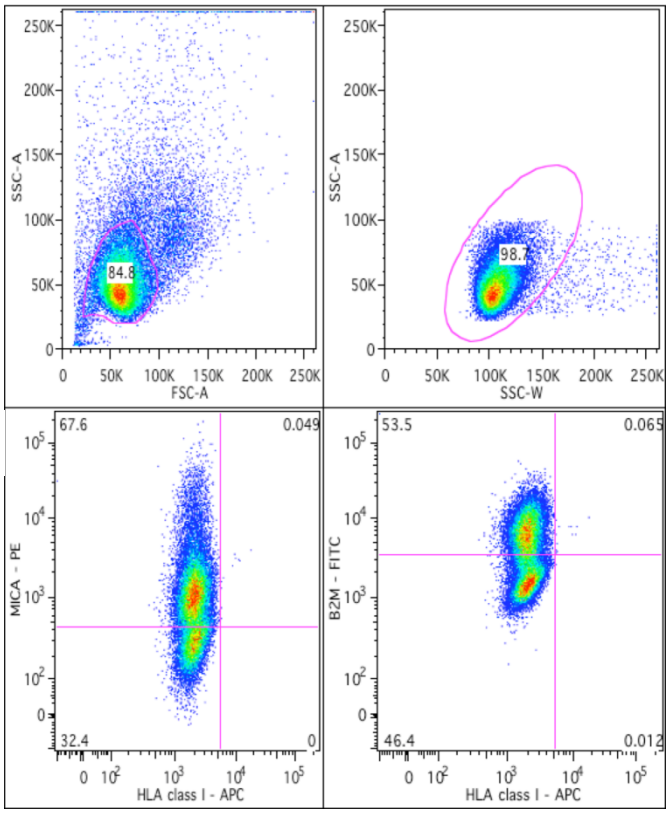
Fragments containing the GOI were digested and amplified with specific primers and run on an agarose gel with 100bp ladder. All three constructs were successfully incorporated into the pcDNA5 vector.



The CHO-cells were then cultured in T175 plates until 80% confluent. Accutase was used to detach the cells, which were then stained with antibodies against HLA-Class I (clone W6/32), MICA and B2M. Unfortunately, HLA-B was not expressed despite multiple attempts to enrich the HLA-B^{+} population using FACS-sorting (Figure 4-9). This lack of expression may have been due to the IRES not functioning as expected.

Figure 4-9. No Expression Of HLA-B (APC) In CHO Cells Transfected With pcDNA5-MICA*009/HLA-B/B2M

Transfected CHO-cells were stained with PE (MICA), FITC (B2M) and APC (HLA-Class I). After gating on cells and excluding doublets, less than 0.1% of cells were APC^{+}. (a) CHO-cells gated, (b.) singlets gated, (c.) 67% expression of MICA but no expression of HLA-B, (d.) 53% expression of B2M, but no expression of HLA-B.



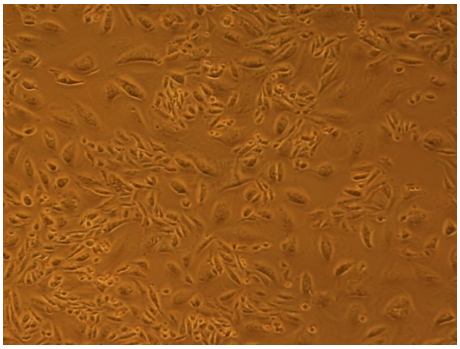
I then opted to create three separate plasmids with three antibiotic resistance genes. First, the CHO-FRT cells were transfected with the pcDNA5-FRT vector with MICA*009 cloned into the MCS, conferring hygromycin resistance (and disrupting zeocin resistance). The CHO-cells were then cultured for a week in media supplemented with hygromycin to ensure all the non-transfected cells were dead. The CHO-MICA*009 cell line was then transfected with a pcDNA3.1(+) plasmid into which a zeocin resistance gene had been inserted, as well as B2M. Unlike the pcDNA5-FRT vector, transfection with pcDNA3.1(+) does not stably integrate into the cell genome, so antibiotic selection was applied for 8 weeks and flow sorted to ensure the CHO-cells expressed both MICA*009 and B2M. Finally, HLA-B*51, B*52 and B*35 were separately cloned into

the pcDNA3.1(+) vector along with a neomycin resistance gene. A neomycin vector control was also produced as an HLA-Null control cell line. These were then transfected into the CHO-MICA*009/B2M cells and the lines were maintained in media supplemented with hygromycin, G418 and zeocin for the remainder of the experiments. Figure 4-10 shows the cells growing well under triple selection.

To ensure the CHO-cells maintained expression of all three constructs the cell lines were periodically flow-sorted. Figure 4-11 shows the four cell lines after flow-sorting. As can be seen, the HLA-Null CHO-cell line expressed over 95% B2M and MICA*009, but <1% HLA. The HLA-B*51, B*52 and B*35^{+} cell lines all expressed over 90% HLA after flow-sorting.

Figure 4-10. Triple Transfected CHO Cells Grow Well Under Multiple Drug Selection

*(a.) Normal morphology of CHO-FRT cells with no antibiotic supplementation. (b.) Morphology of CHO-MICA*009/B2M/HLA-B cells with hygromycin 200ug/ml, G418 650ug/ml and zeocin 100ug/ml*

a. 

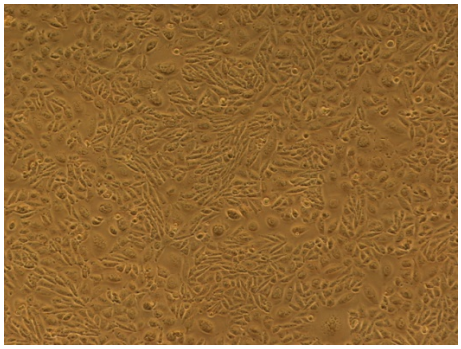
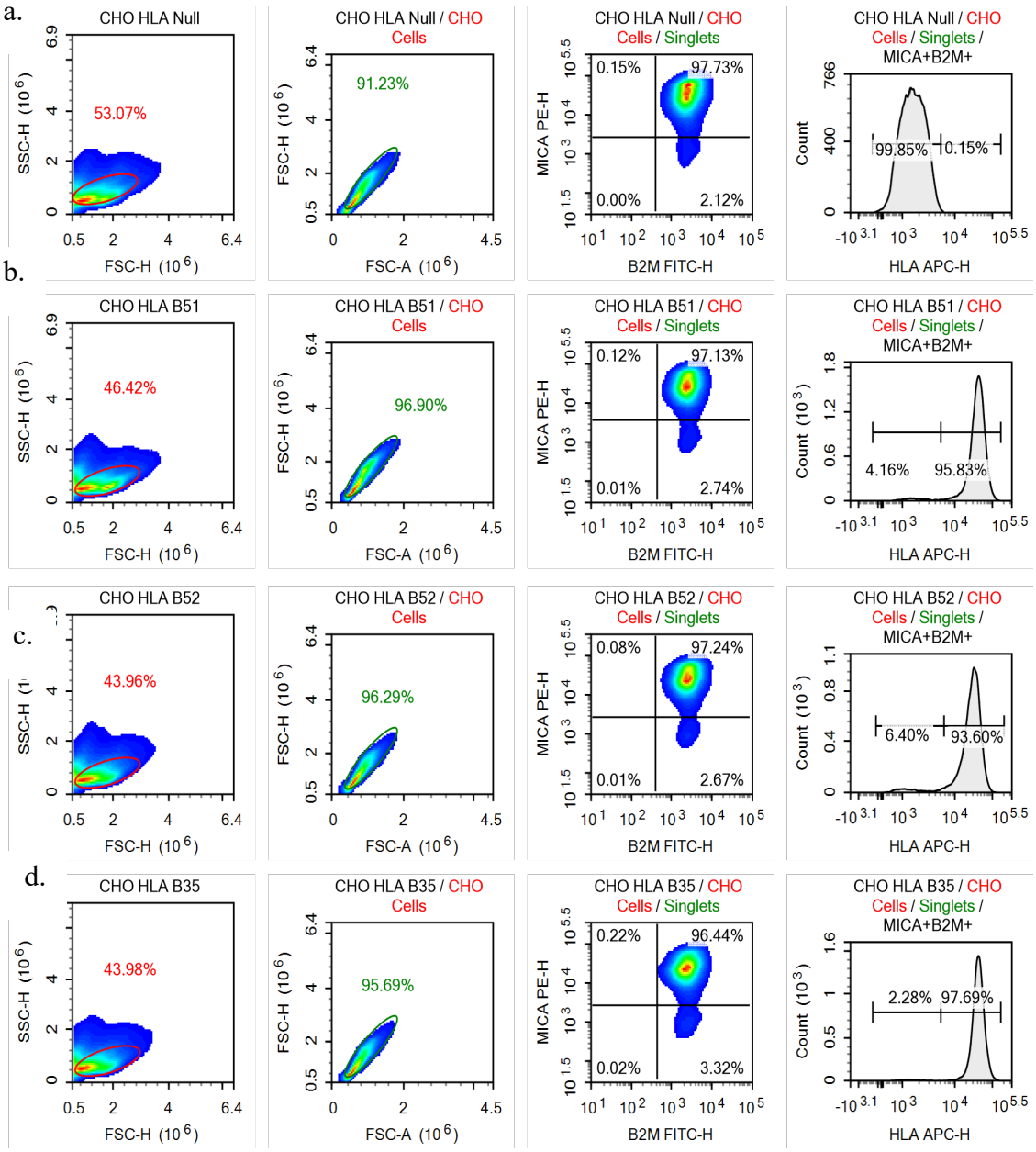
b. 

Figure 4-11. Expression Of Constructs In Triple Transfected CHO-Cell Line.

*After one week of triple drug selection and flow sorting on $MICA^{+}B2M^{+}HLA\text{-}B^{+}$ cells, there was good expression of all constructs. (a). 97% MICA*009, 99% B2M, 0% HLA-B expression in the double transfectant **HLA-Null** cell line. (b.) 97% MICA*009, 99% B2M, 95% HLA-B expression in the CHO-MICA*009/B2M/HLA-**B*51** cells. (c.) 97% MICA*009, 99% B2M, 93% HLA-B expression in the CHO-MICA*009/B2M/HLA-**B*52** cells. (d.) 96% MICA*009, 99% B2M, 97% HLA-B expression in the CHO-MICA*009/B2M/HLA-**B*35** cells. CHO – Chinese Hamster Ovary, HLA – Human Leukocyte Antigen, SSC – Side Scatter, FSC – Forward Scatter, H – Height, A -Area*



4.6.1 MICA*009 And HLA-B Expression In The CHO-Cell Line Reduces Over Time.

Despite culturing in medium with antibiotic supplementation, the CHO-cell lines began to lose expression of the constructs (Figure 4-12). Over 50% of MICA*009 expression and 15-35% of HLA-B expression was lost within 4 weeks.

The cells were therefore re-sorted to achieve over 90% expression of all constructs. I then used three healthy control PBMC with known KIR3DL1/S1 allotypes to test how effective the CHO-cells were at producing a degranulating response. This was measured using CD107a (LAMP-1) expression on the cell surface. CD107a is released as a result of exocytosis of the cytolytic granules following activation of immune cells. Degranulation was inferred from the percentage of $CD107a^{+}$ cells.

CHO target cells were co-cultured with PBMC and stained with anti-CD107a antibody at the start of the experiment. Initially, I used a protocol co-culturing $1x10^5$ target cells with $1x10^5$ NK cells (the number of NK cells present in donors' PBMC was established prior to, but on the same day as, the degranulation assay). Target cells and PBMC were co-cultured in a 5ml polystyrene tube in the presence of anti-CD107a at 37°C with 5% CO_2 (with monensin added one hour into the experiment) for 5 hours, before washing and staining. Using this protocol, the CHO-cell constructs failed to provoke degranulation in the PBMCs. In order to understand why this was not working, I used CFSE, a dye which is rapidly internalised by cells and retained intracellularly to stain the CHO-cells prior to incubation with anti-CD107a antibody. A reduction in CFSE MFI relates to a loss in cell viability (Figure 4-13a). To ensure that this loss of viability was not due to the constructs transfected into the cells, I ran the same assay with the untransfected CHO-FRT cells and found a similar, but less pronounced loss of viability (Figure 4-13b).

Figure 4-12. Effect Of Time In Cell Culture On Expression Of Constructs In The Double And Triple Transfected CHO-Cells Despite Continuous Drug Selection.

*Representative FACS plots taken **4 weeks** after flow sorting to >90% purity of all constructs. The HLA-Null double transfectant shows loss of MICA*009, but not B2M over time. Triple transfectants show loss of MICA*009 and HLA-B, but not B2M over time. (a). 46% MICA*009, 99% B2M, 0% HLA-B expression in the double transfectant **HLA-Null** cell line. (b.) 49% MICA*009, 99% B2M, 65% HLA-B expression in the CHO-MICA*009/B2M/HLA-**B*51** cells. (c.) 49% MICA*009, 99% B2M, 84% HLA-B expression in the CHO-MICA*009/B2M/HLA-**B*52** cells. (d.) 50% MICA*009, 99% B2M, 85% HLA-B expression in the CHO-MICA*009/B2M/HLA-**B*35** cells. CHO – Chinese Hamster Ovary, HLA – Human Leukocyte Antigen, SSC – Side Scatter, FSC – Forward Scatter, H – Height, A -Area*

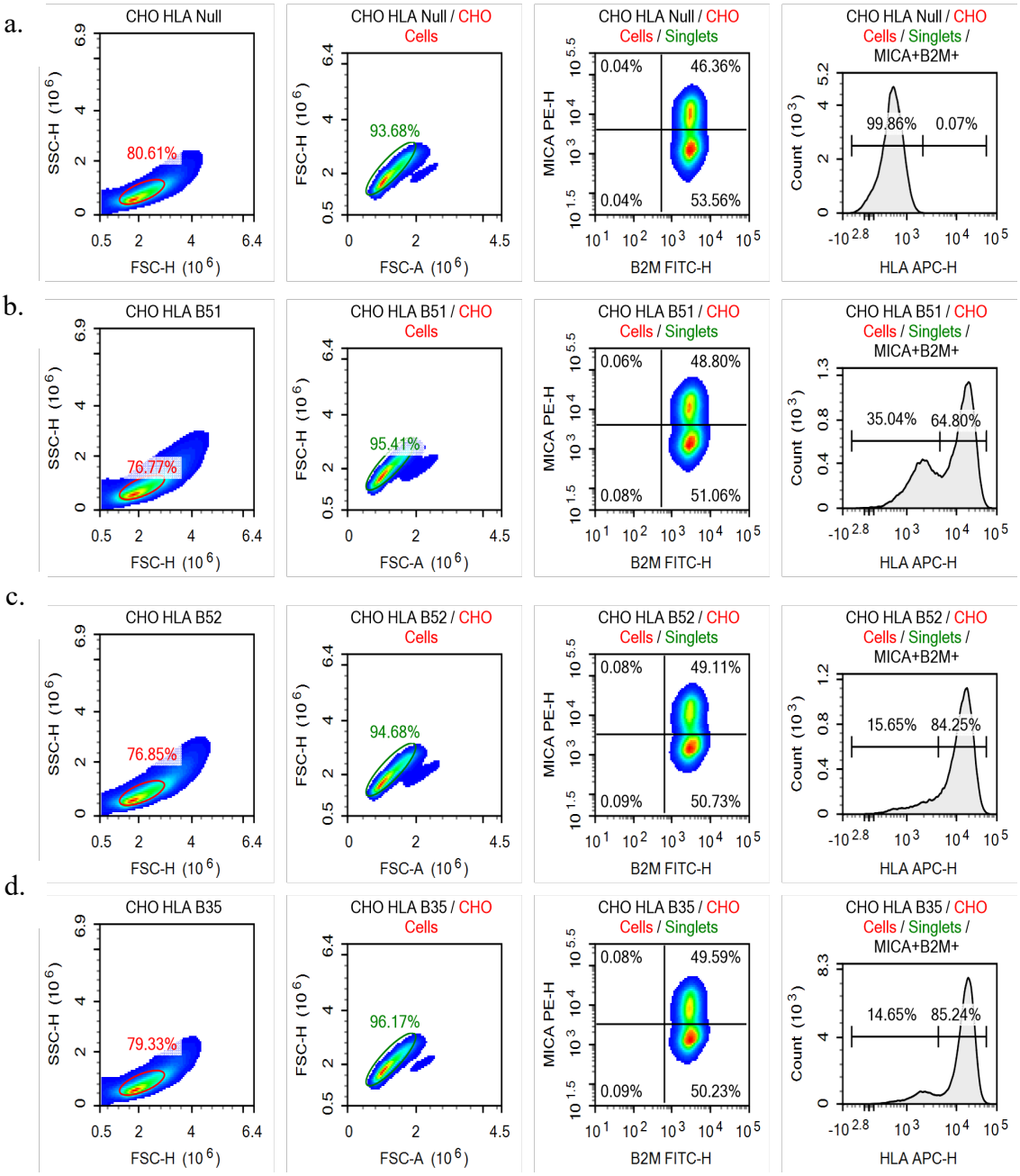
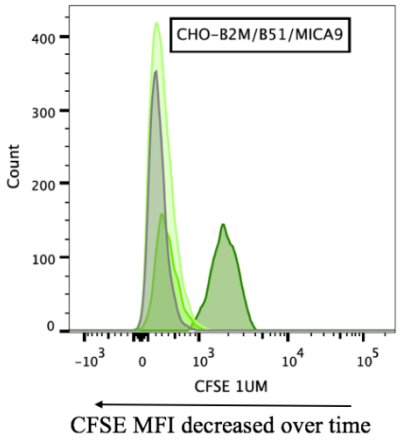


Figure 4-13. Loss Of Expression Of Constructs Over Time In Culture.

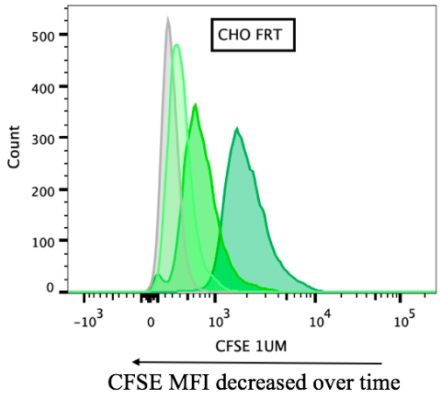
*(a.) CHO-MICA*009/B2M/HLA-B cells and (b.) CHO-FRT cells lose viability in less than three hours of co-culture in a sterile 5ml polystyrene tube. This was thought to be due to the CHO-cells failing to adhere to the coated FACS tubes and resulting a loss of viability.*

a.



	Sample	MFI
	CHO triple transfectant - Unstained	10
	CHO triple transfectant - 1uM CFSE 5 hrs	14
	CHO triple transfectant - 1uM CFSE 3 hrs	16
	CHO triple transfectant - 1uM CFSE 15 mins	200

b.



	Sample	MFI
	CHO-FRT - Unstained	15
	CHO-FRT - 1uM CFSE 5 hrs	30
	CHO-FRT - 1uM CFSE 3 hrs	80
	CHO-FRT - 1uM CFSE 15 mins	200

This loss of viability was likely to be due to CHO-cells failing to attach to the polystyrene FACS tube, while this is not normally a problem for 1 hour at 4C whilst staining with antibodies prior to FACS work, 5 hours at 37C appeared to be too long for the CHO-cells to remain unbound and thus likely resulted in cell death (although this was not tested directly). An alternative method was therefore adopted. The triple transfectant CHO-cells were seeded in 96 well plates and allowed to grow to near confluence. This allowed 5,000

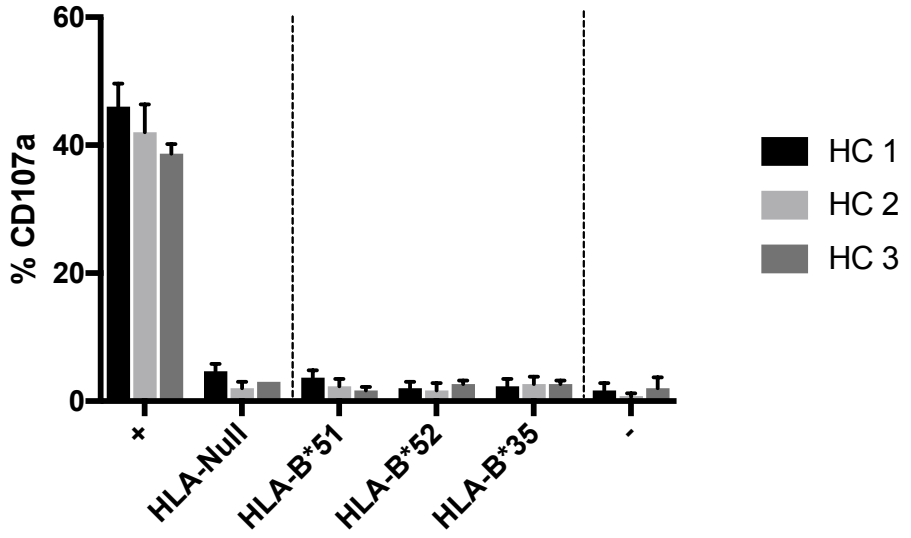
cells to grow per well. I tested this protocol using PBMC from three healthy controls with known KIR3DL1/S1 allotypes. I then added PBMC containing 5,000 NK cells (to maintain a 1:1 ratio) to the well in the presence of anti-CD107a antibody and monensin (after 1 hour). The experiment was run in triplicate (to increase the number of events to 15,000) with three experimental repeats on different dates (using frozen aliquots of PBMC). The cells were then detached, washed and stained for analysis.

Using this protocol, I found that there was measureable degranulation (55%) in PBMCs exposed to the positive control (PMA and ionomycin) but a much smaller response to the MICA*009 control (5%). The triple transfectants (1-2%) provoked minimal CD107a expression as did the negative control (1%) (Figure 4-14a). This level of CD107a expression is far below that reported using non-adherent cells to provoke degranulation. I then attempted to see whether a longer incubation time could improve the response (Figure 4-14b). While there was a small increase in CD107a expression up to 8hrs, the results remained disappointing.

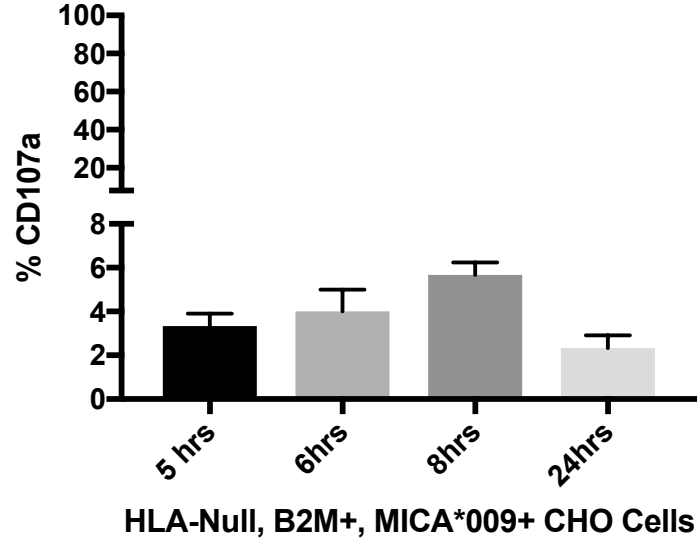
Figure 4-14. CD107a Expression on Peripheral Blood Mononuclear Cells From Three Healthy Control Individuals Following Co-Culture CHO-Cell Transfectants.

(a.) There is good CD107a expression on PBMCs co-cultured with the positive control (PMA + ionomycin), but much less when co-cultured with the HLA-Null cells. The HLA-B triple transfectants and negative control provoked minimal, but similar levels of CD107a expression on the PBMCs. (b.) There was a small increase in CD107a expression if the assay was run for a longer period of time, peaking at 8 hrs. This experiment used PBMC from HC 1

a.



b.



4.7 Creation Of 721.221 B Cell Target Cell Line

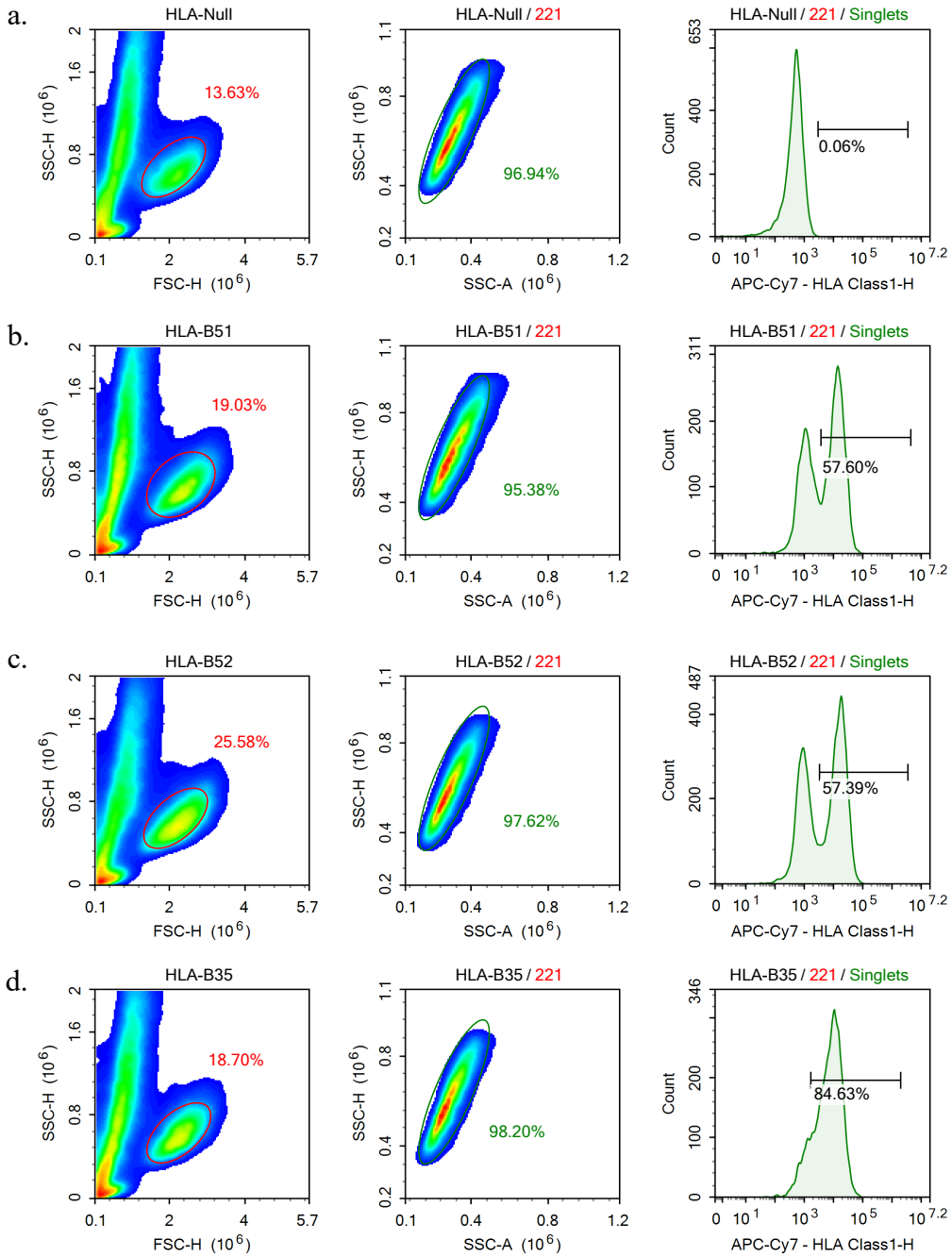
The purpose of creating target cell lines expressing different HLA constructs was to allow differentiation between the inhibitory potentials of various HLA-B-KIR3DL1 combinations. In my hands, the CHO-cell line did not provoke high enough levels of CD107a to make meaningful observations between the constructs.

One possible reason for the poor degranulating response from the CHO-cell line could be a consequence of its adherent nature. Traditionally, CD107a assays are carried out using a non-adherent cell line, allowing cytotoxic cells 360° access to the target cell. As an alternative, I obtained the HLA-null 721.221 cell line (courtesy of the Moffett laboratory, Cambridge, UK) and cloned in HLA-B constructs to use as target cells. The 721.221 (henceforth 221) cell line was formed by Epstein-Barr virus (EBV) transformation of a lymphoblastoid cell line. It is HLA-Class I null, but expresses activating NK receptors, so unlike the CHO-cell line (which required MICA*009 to trigger a degranulating event), there was no need to transfect the MICA construct. Furthermore, there was no need to transfect B2M as 221-cells are human in origin and constitutively express B2M.

I used the pcDNA3.1(+) neomycin vectors with HLA-B*51, B*52, B*35 as well as the vector control to transfect naïve 221-cells. Transfecting B cells is more challenging that CHO-cells and traditional lipid-based transfection methods did not work. I therefore used electroporation to ensure the plasmids were taken up by the 221-cells. After 2 days of recovery, the cells were flow sorted. Figure 4-15 shows the level of HLA expression on 221-cells four weeks after flow sorting. As can be seen, the 221-cells also lose HLA-expression over time, despite continuous antibiotic selection. For this reason, the cells were checked for HLA expression and flow sorted to >90% expression prior to functional assays.

Figure 4-15. Expression Of Genes Of Interest In 221 Cell Constructs Four Weeks After Flow Sorting.

*In each row, cells are gated firstly on 221-cells using FSC and SSC, then on singlets using SSC-H and SSC-A and finally histograms of HLA-Class I staining are shown. (a.) HLA-Null cells have 0% HLA expression. (b.) HLA-B*51 cells express 55.7% HLA-B. (c.) HLA-B*52 express 57% HLA-B. (d.) HLA-B*35 express 85% HLA-B. HLA – Human Leukocyte Antigen, SSC – Side Scatter, FSC – Forward Scatter, H – Height, A -Area*



4.7.1 721.221 Cell Transfectants Provoke A More Profound Degranulation Response Than CHO-Cell Transfectants

To test how effective the 221-cells were at provoking a degranulating response, I used the same healthy control individuals with known KIR3DL1/S1 allotypes to test the cell lines. 1 x10^5 221-cells were co-cultured in a 1:1 ratio with PBMC containing 1x10^5 NK cells (as previously described), Figure 4-16 shows the level of degranulation observed after 5 hours of co-culture.

In addition to provoking a more robust CD107a response from PBMC, the 221 cell line was also easier to use in the assay as the co-culture could take place in a capped 5ml polystyrene tube, which could then be used in downstream cytometric assays. Growing the CHO-cells in 96 well plates until 80-90% confluent was challenging as not all the cell lines grew at the same rate and if they were grown too densely, the assay could not be carried out. In contrast, the 221-cells could be counted and aliquoted on the day of the assay.

4.7.2 Confirming Transfected Constructs Within 721.221 Cells

The antibody used to detect HLA-B throughout this thesis is a W6/36 clone. This clone is unable to differentiate between different HLA-B allotypes. To help ensure that the samples did not become contaminated during passages, they were periodically tested for each HLA-B construct. Primers were designed to amplify unique regions within HLA-B*51, B*52 and B*35. On a monthly basis, DNA was extracted from 221 cell transfectants and screened for contamination. Figure 4-17 shows the size of the amplified

products from each primer pair. Primers were based on Bunce et al. (Bunce et al., 1995) and are used routinely in clinical transplantation laboratories for HLA typing.

Figure 4-16. CD107a Expression On PBMC From Three Healthy Control Individuals Following Co-Culture With 221-Cell Transfectants For 5 Hours

There is robust CD107a expression in PBMCs co-cultured with the positive control and HLA-Null controls. The HLA-B transfectants provoke less CD107a expression on PBMC than their positive control counterparts and negative controls provoke low levels of CD107a expression. Dotted line separates the positive and negative controls from the HLA transfectants. Each experiment was repeated three times.

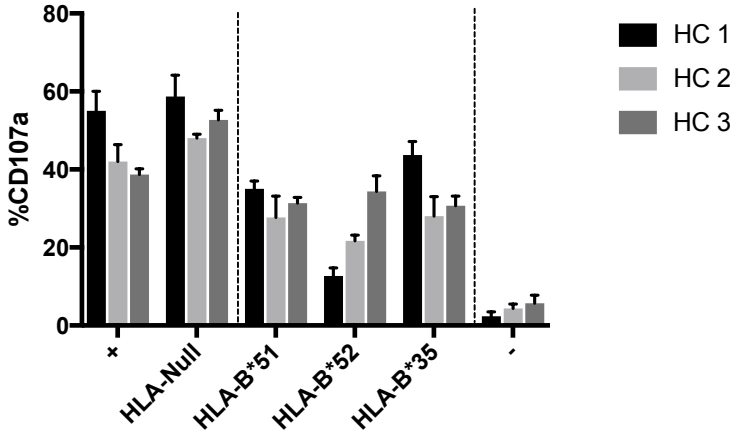


Figure 4-17. Testing For Contamination Of HLA-B Transfectants

*DNA from each transfectant is amplified in the presence of primer pairs 1-(HLA-B*51), 2-(HLA-B*52) and 3-(HLA-B*35). If more than one product was detected when run on an agarose gel, the sample was considered contaminated. In this gel, all three products are shown as well as a vector control.*

4.8 CD107a Expression After Co-Culture With 721.221 Cell Transfectants

4.8.1 CD107a Expression On Natural Killer Cells

Degranulation assays were carried out on nine donor PBMC with the 221 cell line constructs. Experimental repeats were carried out an average of three times (range 2-9) when enough PBMC were available. The percentage of CD107a expressed was adjusted according to the negative control for each sample (Figure 4-18).

The HLA-B*51 cell line appeared to inhibit degranulation more than the HLA-B*52 and B*35 cell lines, however, there were no significant differences in the levels of CD107a expressed by NK cells between the four cell lines (P=0.0736).

4.8.2 CD107a Expression On CD8 T Cells

CD107a expression was examined in the T cell compartment to establish whether CD8 T cells may have an effect in early target cell lysis. Figure 4-19 shows the level of degranulation in CD8 T cells. There was very little CD107a expression in the CD8 T cell compartment. This low level CD107a expression was also seen in the positive control (PMA + ionomycin), with 3.12 ± 1.45 % cells expressing CD107a.

Figure 4-18. Natural Killer Cell CD107a Expression In The HLA-Null, HLA-B*51^{+}, HLA-B*52^{+} And HLA-B*35^{+} 721.221 Cell Lines.

There was no significant difference in CD107a expression on NK cells co-cultured with the three HLA-B transfected cell lines. Expression levels were normalised against a negative control prior to comparison. ns – not significant. Each experiment was repeated three times.

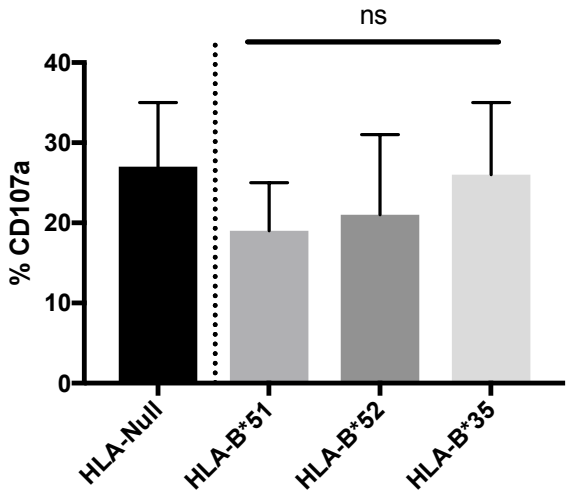
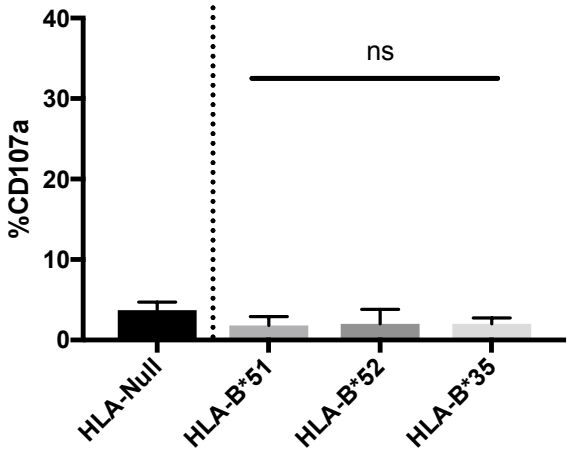


Figure 4-19. CD8 T Cell CD107a Expression In The HLA-Null, HLA-B*51^{+}, HLA-B*52^{+} And HLA-B*35^{+} 721.221 Cell Lines

There was no significant difference in CD107a expression on CD8 T cells co-cultured with the three HLA-B transfected cell lines. Expression levels were normalised against a negative control prior to comparison. ns – not significant. Each experiment was repeated three times.



4.9 Discussion Of Findings

There have been two attempts in the past to establish a link between *KIR3DL1/S1* and BD. In 2007, Middleton et al. examined 93 Turkish cases and 43 controls for the presence/absence of *KIR3DL1/S1*, but did not find any association when controlling for the effects of *HLA-B*51* (Middleton et al., 2007). Recently, Erer et al. used a large and carefully complied dataset of 1,799 Turkish BD cases and 1,710 matched controls to examine the effects of *KIR3DL1/S1* in BD. They found an association between presence of *KIR3DS1* and ocular disease, but no effect of *KIR3DL1/S1* overall on disease-risk (Erer et al., 2016). Our data reflected some of these findings but did not confirm the association between *KIR3DS1* and ophthalmic disease. This may be due to very low power (14%) resulting in a type II error, as there was minimal difference between the BD and HC groups. I found *KIR3DL1* to be present in 96% of BD cases in the UK cohort and 97% in the European Subgroup, whereas *KIR3DS1* was present in 42% of the UK cohort and 45% of the European Subgroup. The lower frequency of *KIR3DS1* may have reduced the power of the study enough to miss an association between ophthalmic disease and *KIR3DS1*. It is also possible that the ophthalmic disease referenced in the paper by Erer et al. may have been phenotypically different that found in the UK cohort (further discussed in Chapter 5).

I did not find any specific allele to increase or decrease the risk of developing BD. Nor did I find an association when alleles were grouped by their inhibitory-potential. The combination of a low-expressing *KIR3DL1* allele and an activating allele (*KIR3DL1*^{LOW}*/KIR3DS1*) was associated with increasing the risk of BD (OR 2.47 in UK cohort and 2.35 in European subgroup). This association survived correction for multiple testing reducing the risk of a type I error and was well powered to avoid a type II error (93%). Of the low-expressing *KIR3DL1* alleles, *KIR3DL1*00501* was the most frequent

(possessed by 82.18% of individuals with BD and 70.78% of HCs in the UK cohort) followed by *KIR3DL1*00701* (possessed by 17.82% of individuals with BD and 25.32% of HCs in the UK cohort). *KIR3DS1* was not found to be polymorphic and only *KIR3DS1*013* was identified in those individuals analysed.

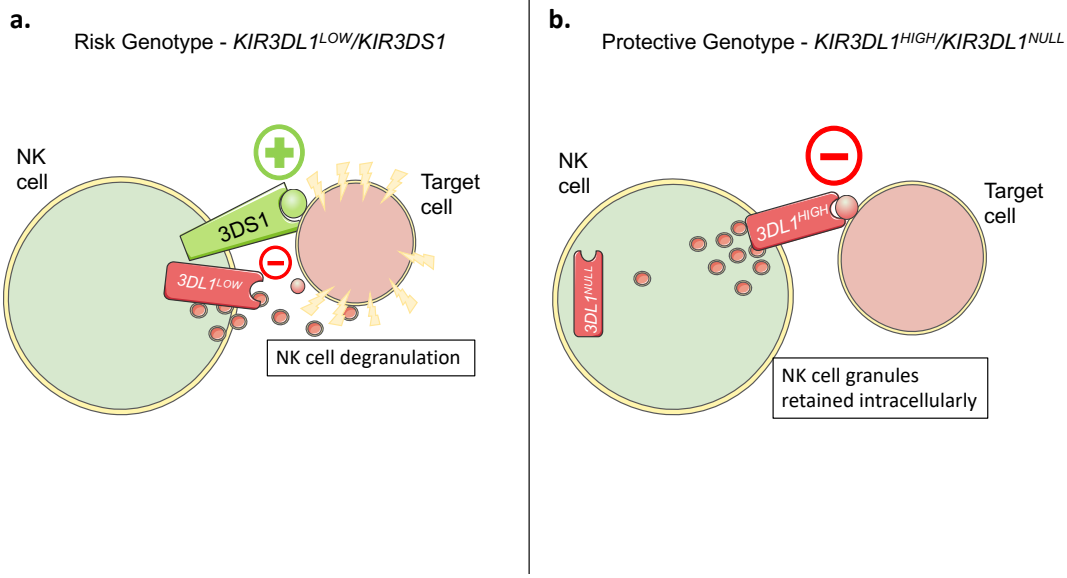
A combination of the high-expressing *KIR3DL1* allele and a null *KIR3DL1* allele ($KIR3DL1^{HIGH}/KIR3DL1^{NULL}$) was associated with decreased risk of BD (OR=0.53 in UK cohort and 0.36 in European subgroup). This association survived correction for multiple testing reducing the risk of a type I error and was well powered to avoid a type II error (92%). Of the high-expressing *KIR3DL1* alleles, *KIR3DL1*0010101* was the only allele implicated in protection from disease and was present in 14.77% of individuals with BD and 16.38% of HCs.

It is possible to explain the increased risk conferred by the $KIR3DL1^{LOW}/KIR3DS1$ genotype in two ways (Figure 4-20). Firstly, the presence of the conserved *KIR3DS1*013* may increase the activation of innate immune cells and drive persistent inflammation. When combined with a weakly inhibitory $KIR3DL1^{LOW}$ allele there may be insufficient inhibition to 'turn off' the inflammatory response. Thus, an individual remains in a pro-inflammatory state, which could be easily exacerbated by a trigger such as a viral infection, trauma or stress causing downregulation of cell-surface HLA and increased cell-mediated cytotoxicity. Similarly, the protective $KIR3DL1^{HIGH}/KIR3DL1^{NULL}$ genotype lacks *KIR3DS1* and relies on other activating receptors to initiate degranulation, although why $KIR3DL1^{HIGH}/KIR3DL1^{NULL}$ should result in protection from disease, while the $KIR3DL1^{HIGH}/KIR3DL1^{HIGH}$ genotype is not implicated in protection, is unclear. It may be that $KIR3DL1^{NULL}$ provides sufficient inhibition during NK cell licencing for other

inhibitory KIRs to be expressed in the presence of their ligand. The role of KIR3DL1*004 is discussed further below.

Figure 4-20. Possible Rationales Of KIR3DL/S1 'Functional Genotypes' In (a.) Risk And (b.) Protection From Behçet's Disease.

(a.) The effects from the activating KIR3DS1 outweighs the inhibitory KIR3DL1 signal. This, combined with potential downregulation of self-HLA in viral infections, leads to degranulation of NK cells and target cell lysis. Uncontrolled and unopposed NK cell activation results in chronic inflammation and local tissue damage. (b.) A strong inhibitory KIR3DL1 signal prevents NK degranulation, thus there is no local inflammation or tissue destruction. This model does not fully explain why individuals who are KIR3DS1 or KIR3DL1^{HIGH} homozygous should not be affected by the same risk and protection effects. It may be that the balancing effect of another KIR3DL/S1 allele is implicated in NK cell education and disease progression.



4.9.1 KIR3DL1*004

The role of *KIR3DL1*004* in disease is of current interest. If the protein is retained intracellularly and therefore is truly 'null', why would it be maintained by natural selection, and be present in ~20% Caucasians? (Martin et al., 2007). Historically, KIR3DL1*004 has been thought to reside intracellularly due to two amino acid

substitutions in the D0 and D1 Ig-like domains (Pando et al., 2003). More recently, Taner et al. published work showing that, while the majority of *KIR3DL1*004* is retained intracellularly, a small proportion is correctly folded and expressed on the cell surface (Taner et al., 2011). This data, complied with evidence that possession of Bw4 and *KIR3DL1**004 slows progression of human immunodeficiency virus (HIV) infection to acquired immunodeficiency syndrome (AIDS) (Martin et al., 2007), make it tempting to hypothesize that KIR3DL1*004 confers some level of inhibitory potential to NK cells that express it. If this is the case, then low surface levels of KIR3DL1*004 could modulate NK cell function in the same way as the KIR3DL1^{LOW} receptor KIR3DL1*005, which is expressed at low levels, but confers robust inhibition to degranulation (Yawata et al., 2006). If KIR3DL1*004 is interacting with Bw4, then it is likely to play a role in educating NK cells and indirectly influencing the proportion of KIR3DL1/S1^{+} NK cells (Morvan et al., 2009).

4.9.2 KIR3DL1*005 And *007

Two low-expressing *KIR* alleles were found to confer risk in combination with *KIR3DS1* (**00501* and *00701*) in this study. In the MC group, there was no effect from *KIR3DL1*00501*, whereas in the ophthalmic group both alleles contributed to disease susceptibility. *KIR3DL1*00701* was found less frequently in both groups, but its effect size was between 5-10x greater than *KIR3DL1*005. KIR3DL1* is broadly represented by three ancestral lineages; *KIR3DS1*01301*, *KIR3DL1*005* and *KIR3DL1*015* (Parham et al., 2011). From these lineages, selection pressures have encouraged an abundance of *KIR3DL1* allotypes to form. The *KIR3DL1*015* lineage is most diverse amongst Africans, whereas the *KIR3DL1*005* lineage is more common in Caucasians (Norman et al., 2007a). While *KIR3DL1*005* has remained stable for more than three million years,

*KIR3DL1*007* segregated as a clade from *KIR3DL1*015* more recently (Parham et al., 2011). It is notable in our cohort that *KIR3DL1*015* was not associated with BD, whereas *KIR3DL1*007* with its narrow binding pattern and *KIR3DL1*005* with its broader specificity are. Despite these biological differences, both receptors are expressed at low levels on the cell surface with KIR3DL1*005 contributing a strong inhibitory response and KIR3DL1*007 a much weaker response (Gumperz et al., 1997; Gumperz et al., 1996; Yawata et al., 2006).

4.9.3 KIR3DS1

KIR3DS1 was found to increase the risk of developing BD in conjunction with $KIR3DL1^{LOW}$ alleles. *KIR3DS1* appeared to be monomorphic in our cohort; *KIR3DS1*01301* being the only allele identified. *KIR3DS1*01301* is thought to be the prototypic *KIR3DS1* allele and is found in all studies populations.

Despite many structural similarities between KIR3DL1 and KIR3DS1, it remains contentious as to whether Bw4 is a ligand for KIR3DS1. There has been one report of KIR3DS1 binding HLA-B*57 (O'Connor et al., 2015) in the context of HIV-1 infection. However, in 2016, HLA-F was identified as a high-affinity ligand for KIR3DS1 (Garcia-Beltran et al., 2016). HLA-F is thought to be the ancestral progenitor of the stress-induced ligand – MICA. Like MICA, HLA-F does not bind peptide, nor require B2M to stably fold and is upregulated during cell-stress (Cerboni et al., 2007; Goodridge et al., 2010).

HLA-F also binds other allotypes of KIR3DL1, but at a lower affinity. In BD, KIR3DS1 may be recognising HLA-F, which has been upregulated due to cell-stress (N. Lee et al., 2010). Our data suggests that this, in conjunction with a $KIR3DL1^{LOW}$ allotype, results in NK cells that are more likely to degranulate than those without an inhibitory KIR3DL1

(i.e. KIR3DS1/KIR3DS1), or indeed an activating receptor (i.e. KIR3DL1^{LOW}/KIR3DL1^{LOW}). KIR3DS1 has also been linked to a number of other diseases without an HLA-Bw4 association (Besson et al., 2007; Trydzenskaya et al., 2013; Venstrom et al., 2010), suggesting that the effects of KIR3DS1 may be independent of Bw4. This is relevant to this study as the *KIR3DL1^{LOW}/KIR3DS1* 'functional genotype' was found to be significant in the Bw6 subgroup as well as the Bw4 subgroup in the UK cohort. However, in the European subgroup the Bw4 group lost its association (Figure 4-1). Suggesting that the 'risk genotype' may be partially (or completely) mediated by mechanisms other than Bw4 interaction. By comparison, the protective KIR3DL1^{HIGH}/KIR3DL1^{NULL} genotype was associated with Bw4 in both the UK cohort and the European subgroup.

In 2017, Carlomagno et al. published findings suggesting that KIR3DS1 may play a role in NK cell education in the same way as KIR3DL1. The authors found that KIR3DS1^{+} NK cell clones could recognize HLA-B*51 when transfected into Bw4- target cells, but not in Bw4^{+} cells. Thus, implying that exposure of KIR3DS1 to Bw4 during early development reduces binding to self-targets later in life.

4.9.4 Expression Of KIR3DL1/S1 In Behçet's Disease

This project aimed to determine *KIR3DL1/S1* allelic variation in BD and to establish whether this variation could explain the role of HLA-B*51 in disease pathogenesis. To do this, a large cohort of individuals with BD and demographically matched HCs were genotyped. Using the data generated, I approached individuals with specific *KIR3DL1/S1* genotypes to donate PBMC for phenotypic and functional analysis. Over a 16-month

period, PBMC from only 22 individuals were collected. There are a number of reasons for this:

1. Individuals who are clinically stable were only returning to clinic on an annual basis.
2. Individuals often required a large number of clinical samples to be taken during their visit and were reluctant to have additional research samples taken, despite donating blood previously.
3. Individuals may not attend clinic or be lost to follow-up.

Of the 22 individuals phenotyped, NK cells made up 5.35% ± 2.31 of circulating lymphocytes. In the past, circulating NK cells in BD have been reported as being raised (F. Kaneko et al., 1985; Suzuki et al., 1992), unchanged (Eksioglu-Demiralp et al., 1999; Saruhan-Direskeneli et al., 2004) and reduced (Hamzaoui et al., 1988; Hasan et al., 2017). However, many of these reports are not comparable due to heterogeneous patient groups, disease activity status, concurrent medications and methodological discrepancies. In 2017, Hasan et al. described reduced numbers of circulating NK cells in the same patient population as that studied here. The authors also found a slight shift toward $CD56^{Bright}$ NK cells compared to healthy controls. Of interest, NK cell percentages were lowest amongst BD cases with active disease and those taking the DMARD azathioprine. Although it did not appear to have a marked impact in the patients studied in our cohort, azathioprine has been previously identified as cause for NK cell depletion (Cseuz et al., 1990; Mellado et al., 2012; Orandi et al., 2017; Pedersen et al., 1986; Prince et al., 1984; Shih et al., 1982). Similarly, our data did not support the finding that there is a significant difference between circulating NK cells in active and quiescent BD.

It is possible that circulating NK cells are being actively recruited to inflamed tissues in BD (particularly during a flare). However, there is no published data specifically investigating the presence of NK cells in BD-lesions. Reduced NK cells in peripheral blood have been described in other diseases, such as type-1 diabetes mellitus (Rodacki et al., 2006), systemic lupus erythematosus (Yabuhara et al., 1996), rheumatoid arthritis (Dalbeth et al., 2002) and psoriasis (Ottaviani et al., 2006). There is further data from the diabetes literature that suggests that NK cells are increased in pancreatic islet cells, thus reinforcing the concept that peripheral NK depletion is coupled with NK cell tissue-aggregation (Gur et al., 2010). Another interpretation of this finding could be that NK cells are depleted in all compartments as a result of BD, or indeed, that a state of NK depletion contributes to BD development. At present, there is insufficient data to discount the hypothesis that NK cells are recruited to inflamed tissues in BD.

KIR3DL1/S1 was labelled with an antibody that recognizes only KIR3DL1 (DX9) and another which recognizes both KIR3DL1 and KIR3DS1 (REA 618). Traditionally, the Z27 antibody clone is used to label KIR3DL1 and KIR3DS1. However, for manufacturing reasons it was not available at the time these experiments were carried out. Low levels of KIR3DL1 were identified on NK cells and practically none on CD8 T cells. Much of the previously published data examining KIR3DL1/S1 allotype binding profiles was carried out on transfected cell lines, so the normal-range of DX9 binding in health and disease is not extensively described and has never been carried out in a cohort of BD patients from the UK before. In 2016, Boudreau et al. published data based on 135 healthy controls and found that the expression level (Low, High or Null) was proportionate to the percentage of NK cells expressing KIR3DL1 (Figure 1-8) (Boudreau et al., 2016). Based on this, it was expected that *KIR3DL1*^{LOW} homozygotes would have a lower percentage of NK cells expressing KIR3DL1 than KIR3DL1^{HIGH} homozygotes. However, the DX9 binding data

shown here did not correlate with the expected binding. For example, 22% NK cells expressed KIR3DL1 in sample 5 (KIR3DL1^{HIGH} homozygote), whereas less than 5% of NK cells expressed KIR3DL1 in samples 1, 2, 3 and 7 (also KIR3DL1^{HIGH} homozygotes). This finding was not dependent on the presence or absence of Bw4 and there did not appear to be a significant effect from medication or disease activity. Similarly, samples 9, 10, 11 and 12 (KIR3DL1^{HIGH}/KIR3DL1^{LOW}) did not express any KIR3DL1 at the time of testing.

A study in 2017, examined KIR3DS1 expression on NK cells of 27 HC donors (Figure 4-21). The authors found that KIR3DS1 was present on ~40% of NK cells using the same staining strategy as that used in this study (Figure 4-22A). They also found no difference in KIR3DS1 expression on cells expressing Bw4I80, T80 or Bw6 (Figure 4-22B). By comparison, I found negligible KIR3DS1 expressed on NK cells in the five individuals possessing *KIR3DS1* (Carlomagno et al., 2017).

CD8 T cells also express KIR. Currently available data suggests that this occurs at a lower level and with less variability than their NK cell counterparts (Bjorkstrom et al., 2012; Ugolini et al., 2001) (Figure 4-22). Furthermore, CD8 T cells appear to frequently express different KIR than NK cells, which may or may not have a self-ligand present. In this project, I determined that expression of KIR3DL1/S1 on CD8 T cells was not correlated to expression in NK cells. However, given the low levels of expression detected, it is difficult to draw any conclusions regarding differences between the individuals tested. The percentage of CD8 T cells expressing KIR3DL1 has been found to vary considerably in different individuals. In 2012, Bjorkstrom et al. examined the expression of KIR3DL1 in 43 individuals and found that KIR3DL1 was expressed on 22.5% NK cells compared to 4% of CD8 T cells; a level of expression comparable to our cohort.

Finally, these results could be compared to those published by Takeno et al, 2004 (Takeno et al., 2004). The authors investigated KIR3DL1 expression (in the absence of data regarding the *KIR3DL1* alleles present) in a group of 40 individuals with BD. The majority (60%) of patients had ophthalmic disease and of the remaining 40% who did not have ocular manifestations, 90% had either neuro-BD or gastrointestinal involvement. The authors also found that KIR3DL1 was reduced on circulating NK cells in BD, but to a lesser extent that in this study (12.99 ± 12.49%). This could be explained due to the severity of BD in the two groups (severe vs less severe), ethnicity or medication. It is also possible that the KIR3DL1 antibody used had a different binding pattern to that used in this study.

Figure 4-21. Gating Strategy For Selecting KIR3DS1$^+$ NK Cell Subsets

KIR3DS1$^+$ NK cells (CD3$^-$CD56$^+$KIR3DS1$^+$KIR3DL1$^-$) in PBMC from healthy controls (A). KIR3DS1 surface expression was evaluated on NK cells from HLA-B Bw4-I80 (n = 12, full squares), Bw4-T80 (n = 7, full circles), or Bw4- (n = 8, full triangles) healthy donors and found to be equivocal across all groups (B). Reproduced with permission from (Carlomagno et al., 2017)

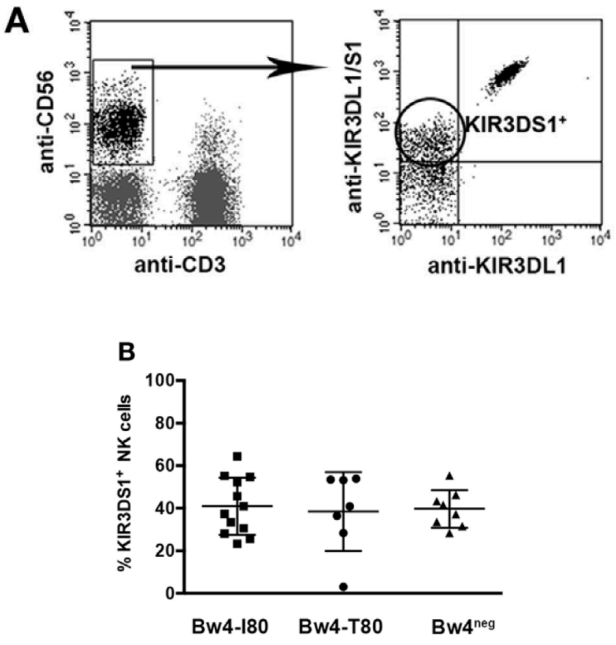
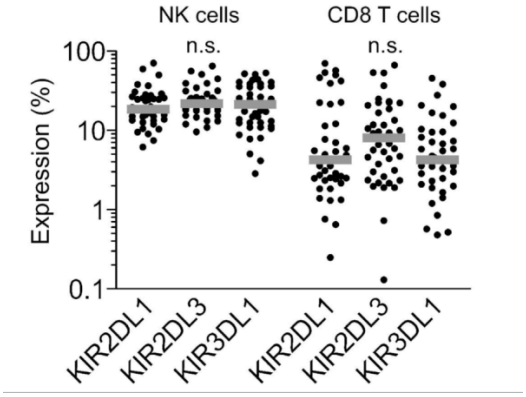


Figure 4-22. Frequency Of KIR3DL1 Expression On NK And CD8 T Cells In 43 Healthy Control Individuals

The authors found KIR3DL1 to be expressed on 40% of NK cells in an HC population. This contrasts with the findings in the project, where KIR3DL1 was expressed on less than 10% of cells in patients with Behçet's Disease. Reproduced with permission from (Bjorkstrom et al., 2012).



4.9.5 Degranulation Assays

HLA-null, 221 target cells were used to create HLA-B*51, HLA-B*52 and HLA-B*35^{+} cell lines. These cell lines did not induce significantly different levels of degranulation in NK cells from KIR3DL1/S1-typed donors. I was only able to test a small proportion of the overall number of patients genotyped and I was unable to compare healthy controls with matched KIR3DL1/S1 genotypes. There may have been a trend to suggest that HLA-B*51 provoked less CD107a expression than the HLA-B*52 and B*35 cell lines (Figure 4-18), however, more samples would need to be run to confirm this trend.

4.9.6 Chapter Summary

Behçet's Disease is a heterogeneous condition. Many patients will only ever experience orogenital ulceration, while others go on to develop blinding uveitis or inflammatory brain disease causing long-standing neurological deficit.

This is the first study to examine *KIR3DL1/S1* alleles in BD. I found that a *KIR3DL1*^{LOW}*/KIR3DS1* genotype was associated with an increased risk of developing BD, whereas a *KIR3DL1*^{HIGH}*/KIR3DL1*^{NULL} genotype was associated with protection from disease. My findings correlate with published data in other inflammatory diseases. In 2016, Ahn et al. examined the frequency of KIR3DL1/S1 allotypes in 203 patients with psoriasis and 111 healthy controls of European descent. Their results suggested that presence of a weakly inhibitory *KIR3DL1* allele increased the risk of developing psoriasis, whereas *KIR3DL1*004* was protective (Ahn et al., 2016). Similarly, Diaz-Pena et al. found that the combination of *KIR3DS1*013* and *KIR3DL1*004* was protective in ankylosing spondylitis (Diaz-Pena et al., 2010). In 2016, a large cohort of African-Americans with multiple sclerosis were tested for any *KIR* associations. The authors found that presence of *KIR3DL1* in conjunction with Bw4 lead to protection from disease, however, these benevolent effects were overridden by the risk attributed from *HLA-DRB1*15:01* (Hollenbach et al., 2016). There are still relatively few disease-association studies investigating the effects of *KIR* allelic variation. The findings from this project need to be repeated in other BD cohorts to confirm their validity.

5 A Prospective Survey of the United Kingdom To Identify The Incidence Of Ophthalmic Manifestations In Behçet's Disease

There is no published data exploring the ocular manifestations of BD in the UK. Nor are there robust statistics available to inform clinicians and patients of the incidence of ophthalmic BD. Knowledge of the incidence of eye disease within the UK is important to better inform patients of their prognosis. Given the heterogeneity of BD across the globe; it is not adequate to counsel patients based on data from another population (Mahr et al., 2008). This information is also important for planning clinical trials, calculating costs to the health service and determining best management strategies.

The ophthalmic complications of BD can be particularly challenging to manage. It has been reported that up to 90% of patients who are not treated will lose all vision in both eyes within four years (BenEzra et al., 1978). As previously mentioned, it is likely that the prevalence of BD in the UK is approximately 2 per 100,000, based on unpublished data collected by the Behçet's Disease Centres of Excellence. The incidence of ophthalmic disease varies across the world from 10-80% (Barra et al., 1991; Chamberlain, 1977; González-Gay et al., 2000; Papoutsis et al., 2006; P. Yang et al., 2008; Zouboulis, 1999; Zouboulis et al., 1997). A study from 1977 carried out in Yorkshire, UK suggested that ocular complications of BD were present in 25% of individuals affected. However, the study involved small numbers and was retrospective in design.

In order to collect incidence data, I approached the British Ophthalmic Surveillance Unit (BOSU). BOSU is part of the Royal College of Ophthalmology and was established to survey the incidence of rare ocular conditions.

5.1 Incidence Of Ophthalmic Manifestations Of Behçet's Disease

Behçet's Disease is known to be most common across the ancient Silk Route and most of the epidemiological data has come from countries that are most affected. In 2001, Bang et al. reported a BD prevalence of 3.2 per 100,000 in South Korea. Of these, 50% (n=320) had eye disease (Bang et al., 2001). Data from Germany suggested that the prevalence of BD in 1997 was 1.68 per 100,000 with between 48%-66% (n=150-210) having BD-related eye disease (Zouboulis et al., 1997). A smaller survey reported the incidence in northwest Spain at 0.66 per 100,000 with 30% having eye disease (n=34 over 5 years) (González-Gay et al., 2000).

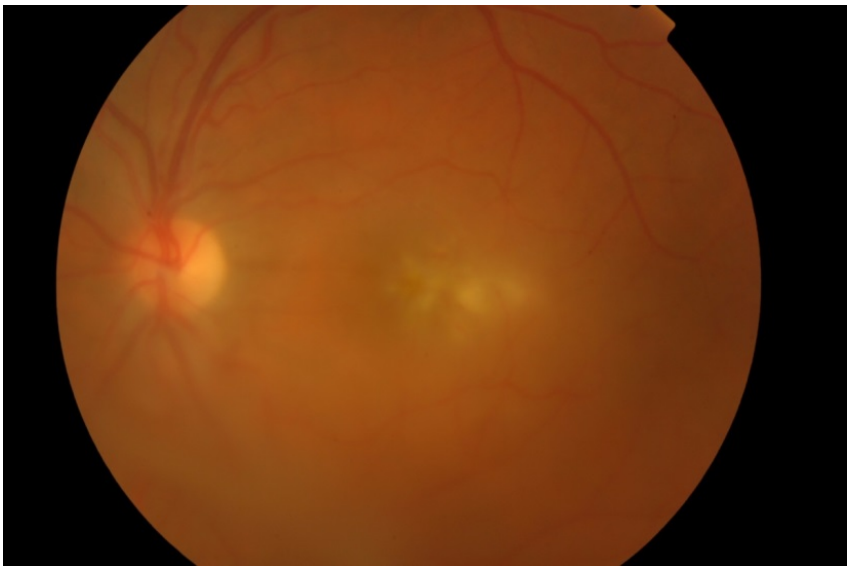
5.2 Case Definition

Patients eligible for the study must have had ocular pathology consistent with BD. Typically, this includes; anterior uveitis, vitritis, vascular occlusion and retinal infiltrates. Rarely, patients may present with episcleritis, scleritis, optic neuritis and ocular surface inflammation.

A retinal infiltrate is a creamy-white lesion, usually larger than a cotton-wool spot and can be found in the posterior pole or periphery. They represent areas of intraretinal neutrophilic aggregation. Figure 5-1 shows a retinal infiltrate at the posterior pole of the left eye.

Figure 5-1. Example Of A Retinal Infiltrate Secondary To Behçet's Disease

Colour fundus photograph of the left eye – there is a retinal infiltrate at the macula with 1+ vitreous haze.



5.3 Reported Cases

Reporting methodology can be found in Chapter 2. Cases were surveyed for 12 months between November 2016 and October 2017. During that time 31 cases were reported via the BOSU yellow-card system. Figure 5-2 details the number of reported cases, correct diagnoses and cases analysed. The study was granted an extension from BOSU in October 2017 to allow a second year of data collection, due to end in November 2018. The results in this Thesis do not reflect those collected during the second year of data collection.

5.4 Incidence Of Ophthalmic Manifestations Of Behçet's Disease

A total of 33 cases of ophthalmic manifestations of BD were reported between October 2016 and October 2017. The location of reporting sites can be seen in (Figure 5-3). There was a good spread of reporting from across the UK, with the exception of the North-East and North-West of England and East Anglia, where no cases were reported.

Figure 5-2. Flow Chart Illustrating The Number Of Cases Reported To The British Ophthalmic Surveillance Unit And The Number Finally Analysed.

Thirty-three cases were reported via BOSU, 29 complete questionnaires were returned and of these, 23 fulfilled inclusion criteria for the study.

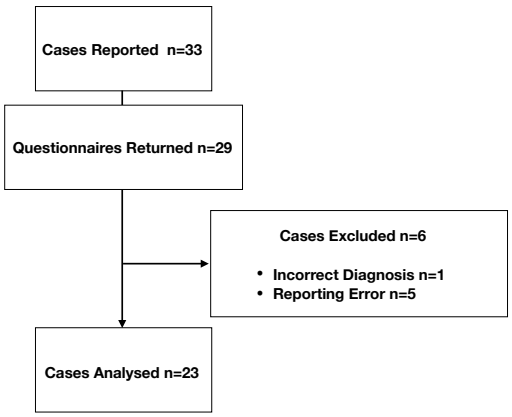
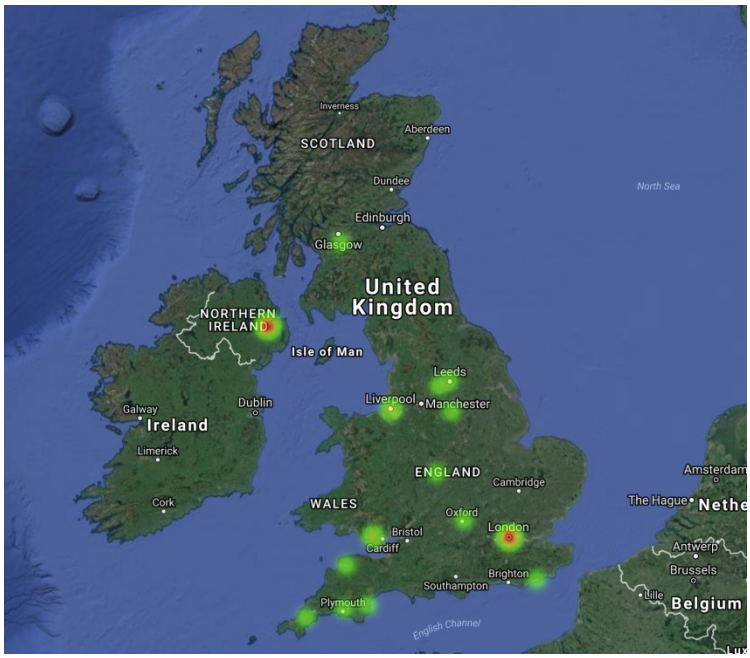


Figure 5-3. Heat-Map Indicating The Locations Of Reported Cases Of Ophthalmic Behçet's Disease

The first-half of patients' post code was collected as demographic data. This was geomapped onto a map of the United Kingdom and shows that most of the reported cases come from areas of high population density.

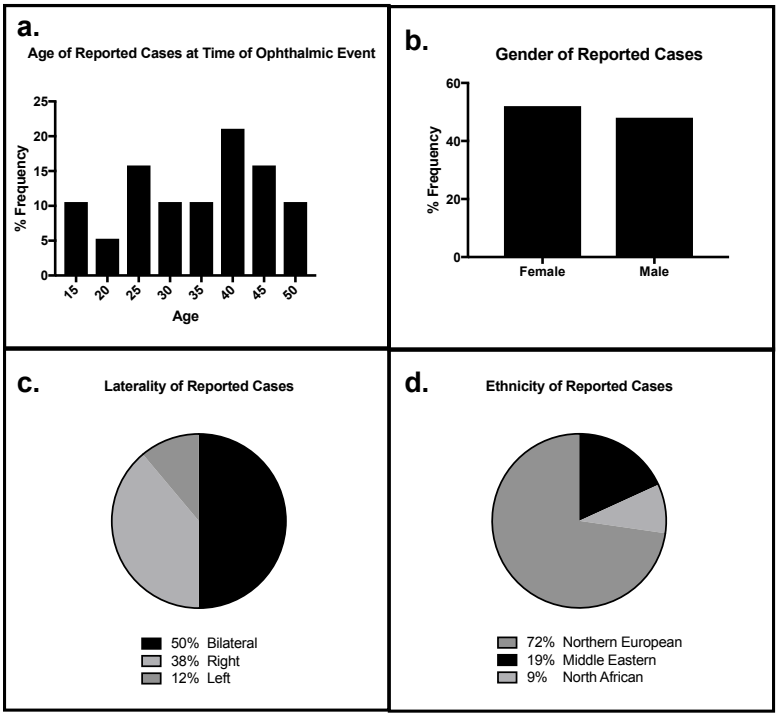


Questionnaires were not returned for four cases; five cases were a reporting error (not the first episode of uveitis) and one case was a misdiagnosis. Over the surveillance period, 23 cases met the inclusion criteria. This gives an estimated annual incidence of ophthalmic manifestations in BD of 0.04 (95% CI 0.02-0.05) per 100,000. In total, 27 eyes were affected. The age, gender, laterality and ethnicity are reported in Figure 5-4. The median age of reported cases was 37±11.1 years (range 15-55 years). There was no predilection for gender, with females making up 52% of cases and males 48%. Fifty per cent of cases had bilateral involvement and 50% unilateral (38% right eye, 12% left eye. The majority of cases were of Northern European ethnicity (72%) followed by Middle Eastern (19%) and North African (9%).

Figure 5-4. Demographics Of Individuals Reported

The age of reported (a.) cases, (b.) gender, (c.) laterality and (d.) ethnicity.

a. Of the cases reported, there was two peaks around ages 25 and 40yrs.
b. There was no gender predominance in those patients with ocular involvement.
c. Half of the cases were bilateral.
d. The majority of cases described were of Northern European ancestry.

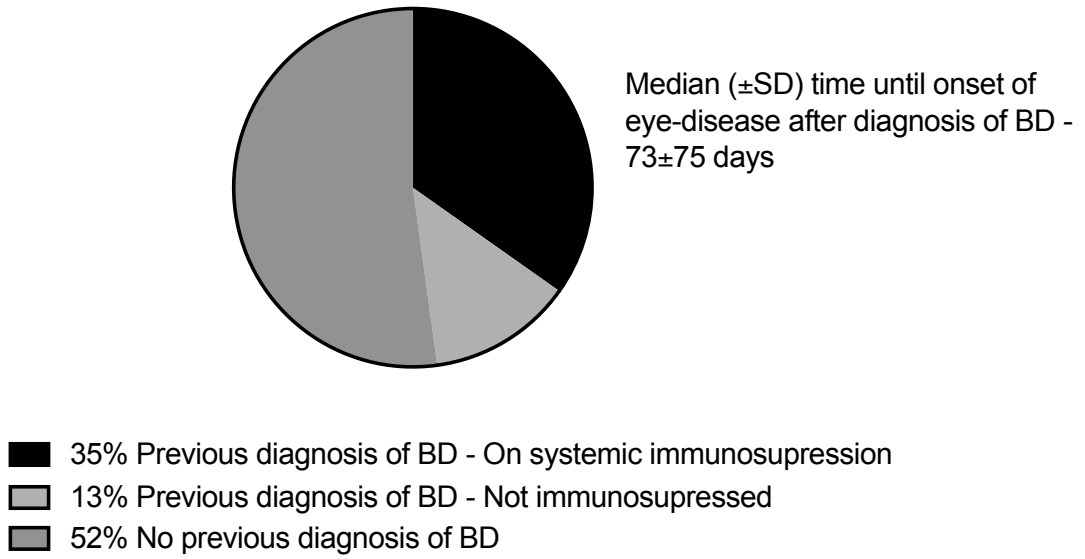


5.5 Clinical Presentation Of Ophthalmic Behçet's Disease

Fifty-two percent (n=12) of individuals were diagnosed with BD at the time they presented with ocular inflammation. The remaining 48% (n=11) of reported cases presented with an ocular inflammatory event after they had a diagnosis of BD (Figure 5-5). Seventy-three percent (n=8) of individuals in this group were taking systemic immunosuppression at the time of their ocular event.

Figure 5-5. Percentage Of Patients With A Previous Diagnosis Of Behçet's Disease

The percentage of patients given immunosuppression is shown along with the median ± SD time until onset of eye-disease. Fifty-two percent of patients did not have an underlying diagnosis of BD, however 48% did, the majority of these were being treated with systemic immunotherapy prior to the onset of eye disease.



5.5.1 Systemic Manifestations Of Behçet's Disease

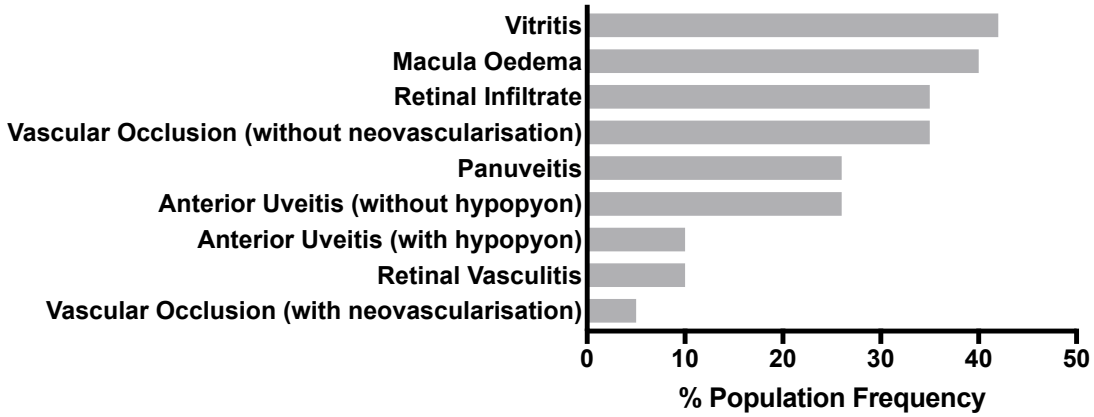
Ninety percent (n=21) of cases had recurrent oral aphthous ulcers, 55% (n=12) reported genital ulceration, 50% (n=11) skin lesions, 10% (n=2) systemic vascular occlusion and 5% (n=1) inflammatory arthritis.

5.5.2 Ophthalmic Manifestations Of Behçet's Disease

All eyes had intraocular inflammation. There were no reported cases of episcleritis, scleritis, optic nerve pathology or ocular surface disease. Anterior uveitis alone was present in 26% (n=5) and in conjunction with a hypopyon in 5% (n=1). Vitritis was reported in 42% (n=8) and panuveitis in 26% (n=5). Cystoid macula oedema (CMO) was present in 40% (n=9) of individuals and retinal infiltrates in 35% (n=12). Inflammatory retinal vascular occlusion was reported in 35% (n=9). Retinal vasculitis was present in 10% (n=2). Ocular Manifestations can be seen in Figure 5-6.

Figure 5-6. Ophthalmic Phenotype At Presentation

The most common ocular manifestations were vitritis, macular oedema, retinal infiltrates and vascular occlusions.



Respondents were asked to specify the cause of visual loss (if relevant). Figure 5-7 shows the top three causes of visual loss (CMO, retinal ischaemia and vitritis). The greatest visual morbidity in BD was caused by CMO (P=0.0102). The mean LogMAR best-corrected visual acuity (BCVA) for those presenting with CMO was 1.1±0.58. This was followed by retinal ischaemia (mean LogMAR BCVA of 0.33±0.12) and vitritis (mean LogMAR BCVA 0.20±0.16). In order to analyse mild and severe ocular inflammation, I

scored ophthalmic manifestations depending on severity and potential threat to vision (Table 5-1). I found no difference between males and females with regard to disease severity (*P*=0.6698). Patients taking corticosteroids alone or one DMARD plus a corticosteroid had more severe ocular manifestations that those taking multiple DMARDs or a biologic medication (*P*=0.0357).

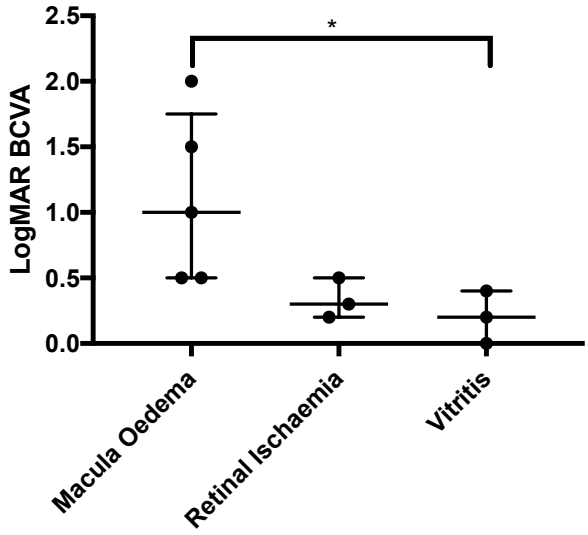
Table 5-1. Scoring Severity Of Ophthalmic Disease

A score 1 was given to intraocular inflammation alone. Any macula oedema, retinal infiltrate or vein occlusion scored 2 due to the increased severity and increased risk of visual loss.

Ophthalmic Manifestation	**Score**
Anterior Uveitis	1
Vitritis	1
Panuveitis	1
Retinal Infiltrate	2
Vascular Occlusion	2
Macula Oedema	2

Figure 5-7. Top Three Identified Causes Of Low Vision

*Individuals with macula oedema presented with significantly worse vision than those with vitritis alone. The mean ± standard deviation LogMAR best corrected visual acuity (BCVA). *P<0.05*

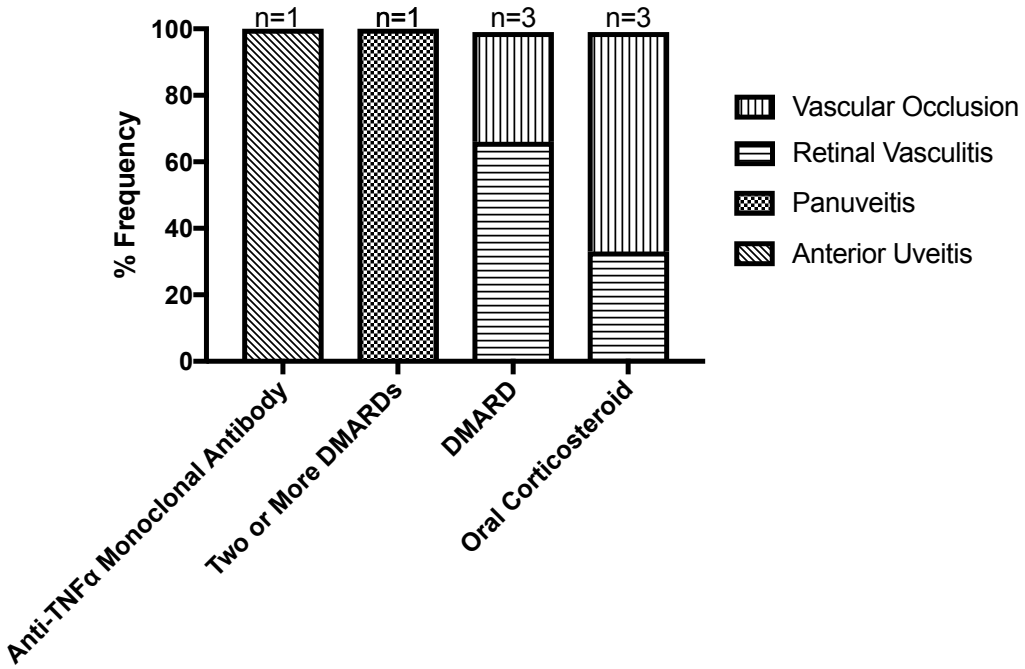


5.5.3 Medical Management Of Patients Prior To Onset Of Ophthalmic Behçet's Disease

Thirty-five percent (n=8) were treated for BD prior to developing eye disease. Of these, 38% (n=3) were taking oral corticosteroids alone, 38% (n=3) were taking oral corticosteroids plus one disease modifying anti-rheumatic drug (DMARD), 4% (n=1) were taking multiple DMARDs and 4% (n=1) were taking an anti-TNFα medication. Figure 5-8 shows a breakdown of ocular manifestations by medication

Figure 5-8. Ocular Manifestations Of Behçet's Disease Occurring In Those Patients Already Taking Immunosuppression

Eight patients were already being treated with a systemic therapy prior to onset of eye disease. Of these, one patient was taking an anti-TNF α monoclonal antibody and one was taking two DMARDs. DMARD – Disease modifying anti-rheumatic drug, TNFα – tumour necrosis factor α.

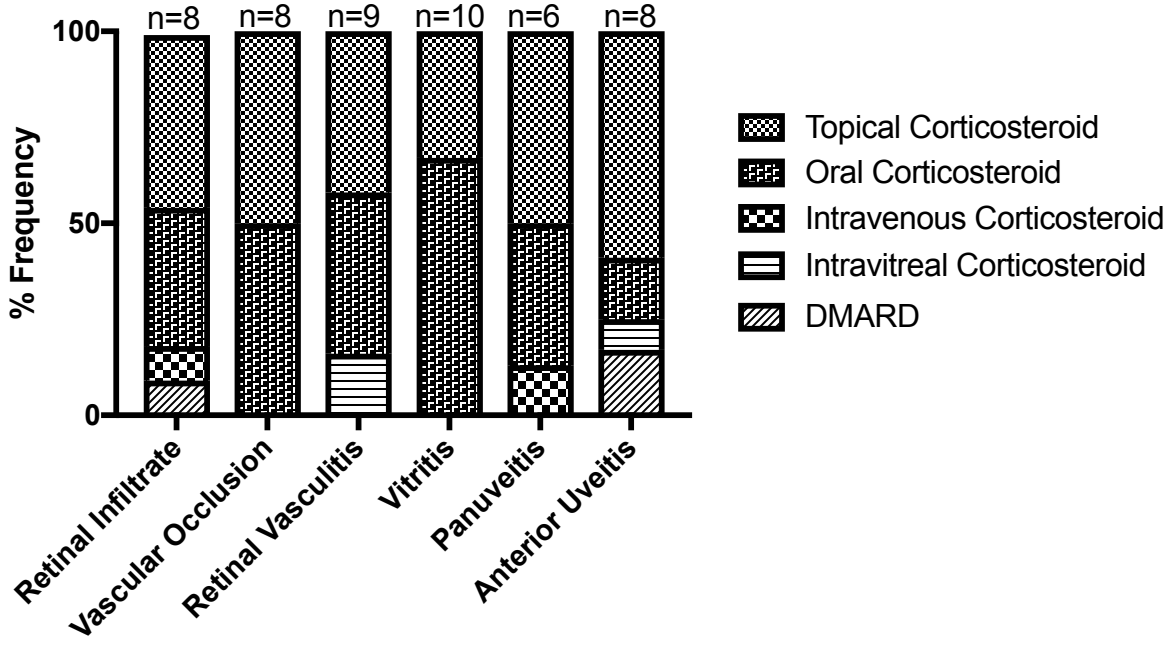


5.5.4 First Line Therapy For Ocular Manifestations Of Behçet's Disease

I asked respondents to state which first-line therapy was given to the patients. Figure 5-9 shows medications prescribed for each indication by percentage. Over half (56%, n=15) of eyes were prescribed topical corticosteroids for both mild and severe disease (see Table 5-1). Twenty-six percent (n=7) of eyes were prescribed oral corticosteroids, 7% (n=2) were prescribed intravitreal corticosteroids, 7% (n=2) were prescribed a DMARD and 4% (n=1) were prescribed intravenous corticosteroids.

Figure 5-9. First-Line Medication Given To Patients With Differing Ocular Inflammation

There was no clear pattern in management of eye disease. Patients with sight-threatening disease were topical steroids and patients with mild anterior uveitis were started on DMARDs. Only three patients were started on a DMARD at the time of presentation, despite EULAR guidelines, 2016. DMARD – Disease modifying anti-rheumatic drug. EULAR – European League Against Rheumatism.



5.6 Discussion Of Findings

Prospective surveillance studies rely on accurate and reliable reporting. There are currently 1420 consultants and associate specialists receiving BOSU reporting cards (personal communication, RCOphth). Typically, 70-75% of cards are returned each month (either to report a case or to indicate that there is 'nothing to report'). Furthermore, 85-90% of individuals will return their reporting cards 6 or more times a year. However, despite good return rates, missing a few cases per year can significantly impact an incidence calculation. A case may be missed, if the patient was managed by a non-consultant or associate-specialist grade doctor (who do not receive reporting cards), or if the reporting doctor was unable to remember seeing a patient at the end of a clinic. There is also the phenomenon of 'reporting fatigue'. This can happen in busy centres where many cases are being surveyed. In this scenario, reporting physicians can be overwhelmed by the number of conditions they are supposed to report and cases can be missed.

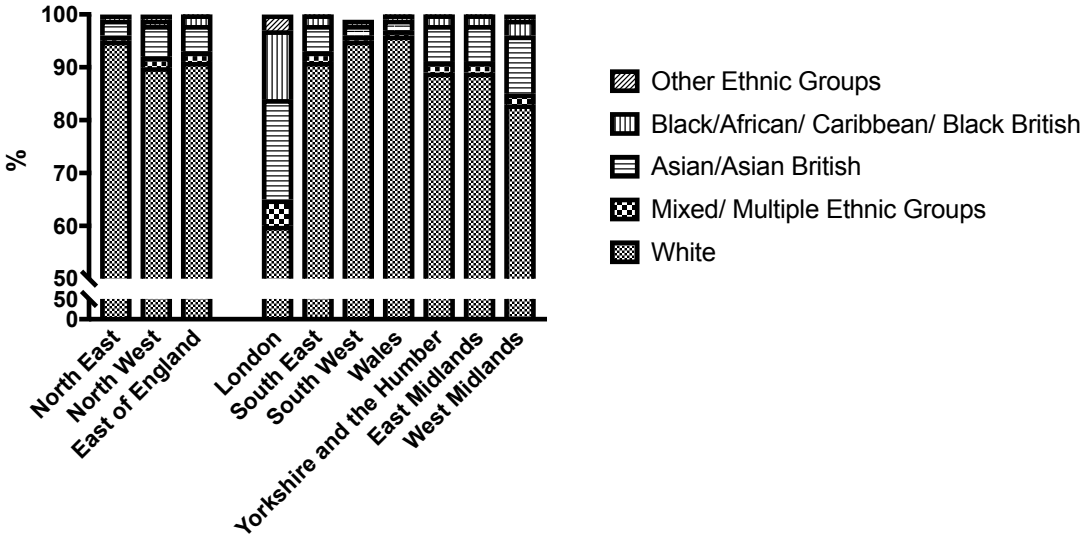
It is important that questionnaires are sent out to reporting clinicians as quickly as possible, so clinical notes are available and accurate information is obtained. In this study, all questionnaires were sent within a week of notification. If no reply was received after two weeks, a reminder was sent, followed by a phone call 1 month later. Four questionnaires were not returned despite our best efforts to obtain the data. These were all from the same centre.

Over-reporting of cases can occur if there is duplicate reporting. This may be due to a patient being referred from a district general hospital to a tertiary referral centre and the case being reported twice.

I did not receive any duplicate reports in this study. There was unequal reporting across the UK. No cases were reported from the North-East, the North-West of England or East Anglia. The last ethnicity data collected by the office of national statistics (ONS) in 2011 found that these areas had relatively few individuals who identified as non-White, which may have contributed to low reporting (ONS, 2011) (Figure 5-10). These regions also have an older population (see below).

Figure 5-10. Self-Reported Ethnicity Data From The National Census Collected In England And Wales, 2011 (ONS, 2011).

Outside of London, over 80% of individuals self-report as 'White'. The three regions on the left did not report any cases via BOSU. The regions on the right of the graph all had at least one case reported.



Finally, the denominator used for incidence studies is typically the population of the region surveyed. In this case, the population of the UK is 65.5 million. However, BD-related eye disease is very unlikely to occur in patients over the age of 50 years. The ONS has estimated that in 2016, 63.1% of the UK population was between 16-64 years. This would reduce the denominator down to 41.3 million and the increase the incidence to 0.056 per 100,000 individuals.

There are a number of problems with this figure. Firstly, in order to be comparable to other populations, it is important that the denominator remains a predictable figure (i.e. the population). Secondly, the proportion of individuals under 16 years or over 64 years varies depending on location (Figure 5-11). For example, in 2016, 31% of the population of North Norfolk was over 64 years, compared to 6% in Tower Hamlets. It is also notable that the areas of the UK with 20-40% of the population over 64 years, such as the North East of England and East Anglia, reported no cases. While ethnicity is important in the pathophysiology of BD; the number of individuals presenting with ocular involvement is small. It is therefore likely that reported cases are coming from areas of high population density and can be described by the Poisson distribution.

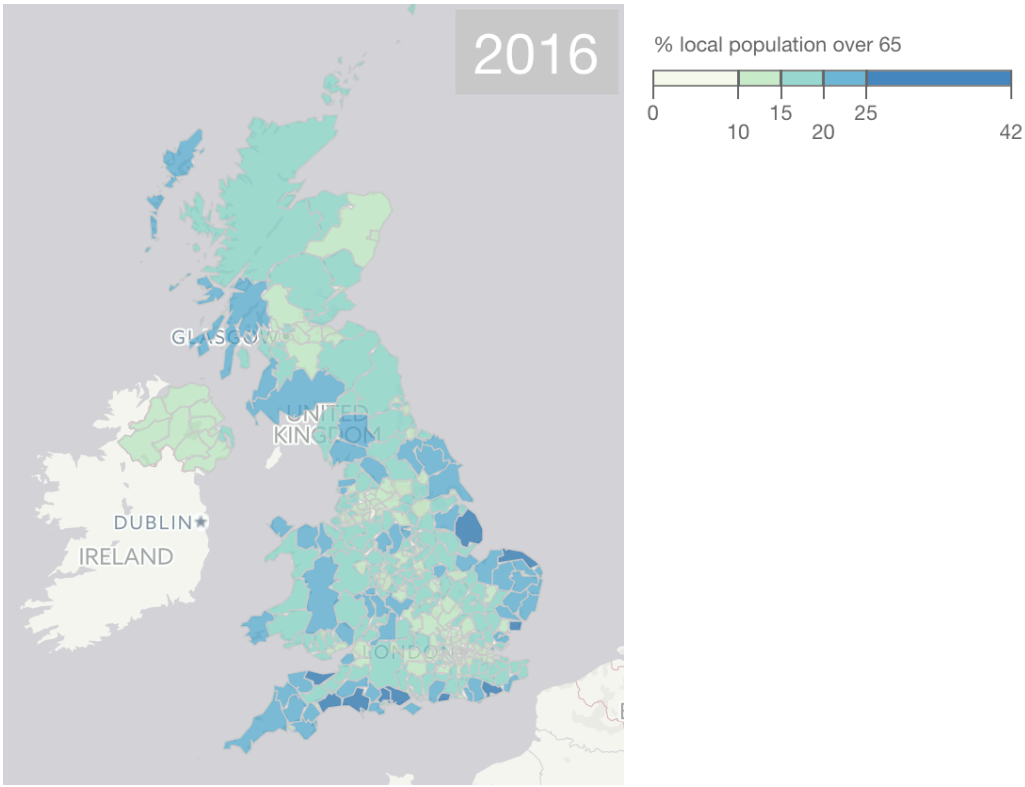
This is the first study to prospectively survey the incidence and manifestations of ophthalmic BD in the UK. The data indicate that the incidence of ocular inflammatory disease in BD is 0.04 per 100,000 individuals. There is no published data to directly compare our findings against because previous studies examining the incidence of BD have used a retrospective methodology to capture all the cases reported within a certain area in a specific time-frame. Similarly, no previous study has examined the incidence of BD-related eye disease.

The incidence of ophthalmic disease in other populations can be calculated using the local incidence of BD and estimating the percentage likely to have ocular involvement. There is incidence data available from Sweden (0.2/100,000) (Mohammad et al., 2013), Italy (0.24/100,000) (Salvarani et al., 2007), the USA (0.38,100,000) (Calamia et al., 2009), Germany (1.0/100,000) (Altenburg et al., 2006) and Korea (2.6/100,000) (Y. B. Lee et al., 2017). Ophthalmic disease occurs in 20-50% of patients with BD, depending on ethnicity (Bang et al., 2001; González-Gay et al., 2000; Zouboulis et al., 1997).

Figure 5-11. United Kingdom Population Aged 65 And Over And The Old Age Dependency Ratio By Local Authority, 2016. (ONS, 2017)

Regions with the highest proportions of individuals over the age of 65 were concentrated around the coast in Lincolnshire, East Anglia, Sussex, Hampshire, Dorset and Devon. These areas reported few cases.

Data exported from ONS, 2017 interim census.
www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationestimates/bulletins/annualmidyearpopulationestimates/mid2017.



The incidence of ocular disease would, therefore be 0.04-1.3/100,000 in these populations: higher than that found in the UK in this study.

Twenty-six percent of individuals identified in this study were either Middle Eastern or North African. In the last national census, 8.8% of the UK identified as 'Black/ African/Black-British/Caribbean' or 'Asian/Asian-British' (ONS, 2017). I found three times more non-'White British' individuals than one would expect from the population of

the UK. This is not a surprising finding, given the increase in frequency of BD across Central Asia, the Middle East and North Africa.

The literature suggests that ophthalmic disease is more common in males and is associated with a more severe disease course (Yazici et al., 1984). I found no difference between males and females in incidence or severity of uveitis. Fifty-two percent of reported cases were diagnosed with BD at the time of presentation. Forty-eight percent already carried a diagnosis of BD and presented with an ophthalmic manifestation between 2-148 days after the diagnosis was made.

I analysed the systemic medication taken by 73% of individuals already carrying a diagnosis of BD. Of note, 38% of individuals were taking oral corticosteroids alone, a further 38% were taking a DMARD as well as corticosteroids, one individual was taking multiple DMARDs and one was taking infliximab (an anti-TNF monoclonal antibody). The patients taking infliximab and multiple DMARDs presented with less severe disease than those taking one DMARD or corticosteroids alone.

Respondents were asked to identify the major cause of visual morbidity in patients with poor vision. Macula oedema was significantly more likely to cause poor vision than vitritis, but not retinal ischaemia. Identifying the major cause of visual morbidity is important for focussing treatment strategies. However, it can be challenging, as more often than not, there are multiple causes for poor vision and the components of ocular inflammation do not occur in isolation. For example, retinal ischaemia may lead to macula oedema. In this situation, it is challenging to identify the major cause of visual loss as both pathologies will be contributory.

Finally, I analysed the first-line treatment given for the reported cases. Topical and oral corticosteroids were the mainstay of treatment for all manifestations. Intravenous corticosteroids were given to one patient with a retinal infiltrate and panuveitis. Intravitreal corticosteroids were given to three eyes of two patients. One patient had bilateral injections for retinal vasculitis and panuveitis and one patient had a unilateral injection for macula oedema. Disease modifying anti-rheumatic drugs were started in two patients. One with bilateral anterior uveitis and one with a retinal infiltrate. The EULAR guidance, published in 2008, was updated in 2016 and is awaiting formal publication. The guidance recommends that all patients with ophthalmic disease are started on azathioprine in combination with corticosteroids. If this does not control the inflammation, patients should be escalated to infliximab, interferon α or ciclosporin (Hatemi et al., 2008). Two individuals were started on a DMARD with corticosteroids as a result of their eye disease. A further 5 were already immunosuppressed, leaving 75% of patients who (n=16) may not have been managed in accordance to these guidelines. There are a number of possible explanations for this. Firstly, respondents were asked for their first-line treatment – it is reasonable to assume that starting a DMARD would not be considered 'first-line' by many clinicians. Secondly, reporting ophthalmologists may have been waiting for a medical opinion before starting immunosuppression or the questionnaire may have been filled in before this had occurred. Finally, patients may have been unable to take azathioprine due to a low thiopurine-methyltransferase level and were either due to start another second line agent or were waiting to be referred to the BD-centres of excellence for a biologic medication.

5.6.1 Chapter Summary

This is the first prospective study to analyse the incidence of ophthalmic involvement in BD. I found the incidence of BD-related ophthalmic disease to be is 0.04 (95%CI 0.02-0.05) per 100,000. Knowledge of incidence and the affected demographics is helpful for clinicians working with BD patients in the UK as it allows us to offer patients a more informed prognosis as well as inform recruitment to future clinical trials.

6 Discussion, Limitations and Further Work

6.1 Discussion Of Thesis

In this PhD, I investigated the genetic associations in a cohort of individuals with BD from the UK. I was able to confirm *HLA-B*51* as a risk factor in this group. *MICA*009* was also associated with BD in this cohort. Of note, I did not find an association between the MICA A6 triplet repeat, a finding that has been confirmed in a number of other populations.

A putative mechanism by which HLA-B*51 may function to influence disease is via its interaction with KIR3DL1. To investigate this, I used sequence-based typing to analyse *KIR3DL1/S1* allelic variation in BD and HC groups. I did not find any specific *KIR3DL1/S1* allele to be associated with BD, however, when 'functional genotypes' were constructed, I found a strong association between possession of a $KIR3DL1^{LOW}/KIR3DS1$ genotype and risk of developing BD. Similarly, I found a protective effect from possessing a $KIR3DL1^{HIGH}/KIR3DL1^{NULL}$ genotype. It appears that these effects are not entirely mediated via Bw4, as the 'functional genotypes' were also found in those lacking Bw4. It may be that there is a partial effect from Bw4 and that presence of the HLA-F OC (which was not examined in this project as it was only confirmed as a ligand for KIR3DS1 in 2016) plays an important role in the $KIR3DL1^{LOW}/KIR3DS1$ risk genotype.

In the UK cohort, both the risk and protective genotypes were significant in individuals bearing the Bw4 epitope. However, in the European subgroup, the risk effect was not statistically significant. The protective effects were significant in the Bw4 subgroups in both the UK cohort and European subgroup. Neither *KIR3DL1/S1* 'functional genotype'

was significant in the *HLA-B*51*$^{+}$ subgroups, which may have been due to small numbers in that particular subgroup.

I then analysed the effects of the risk and protective genotypes in two different clinical manifestations of BD: mucocutaneous disease alone and mucocutaneous disease with ophthalmic involvement. I found that the risk genotype was only significant in the ophthalmic group, whereas the protective genotype was only significant in the MC group. Behçet's disease is a heterogenous condition made up of a constellation of clinical manifestations. There have been reports suggesting that individuals with ophthalmic disease have a more severe disease form than those with MC disease alone (Gul et al., 2012; Yazici et al., 1990). This issue remains contentious. However, if ophthalmic disease represents one end of the disease spectrum, then it may explain why different *KIR3DL1/S1* genotypes are associated with this pathology. A similar rationale has been used to explain the increased prevalence of *HLA-B*51* in patients with ophthalmic disease vs non-ophthalmic disease in the past (L. Krause et al., 2009; Sant et al., 1998), although this is also disputed. In 2001, Gul et al. reported a series of Turkish individuals with BD. The authors used a modified version of the BD total activity index (Yazici et al., 1984) to score disease severity. They then examined *HLA-B*51* presence/absence and heterozygosity/homozygosity in the different groups and found minimal differences between individuals with mild, moderate and severe disease (Gul et al., 2001). Whether these findings can be extrapolated to a UK population in which *HLA-B*51* is less frequently possessed remains unclear.

This project was designed in 2014. The emphasis was to investigate the KIR3DL1 - Bw4 interaction (and by extension the KIR3DL1 - HLA-B*51 interaction) in BD. At that time, the primary ligand for KIR3DS1 was unknown (Burian et al., 2016; Garcia-Beltran et al.,

2016). Similarly, much of the work investigating *KIR3DL1* copy number variation was in its infancy (Tao et al., 2014; Vendelbosch et al., 2015). It would have been useful to have added *HLA-F* into the NGS panel along with all of the *KIR*s so copy number variation could have been established. This may have allowed more detailed interpretation of the KIR3DL1 'functional genotypes' constructed.

In order to create a more homogeneous group of patients, I aimed to control for ethnicity as much as possible. In addition to taking a detailed family history, I ran a panel of AIMs specific for European ancestry. This panel enabled us to create a subgroup of individuals of European extraction who could then be used to confirm genetic associations found in the whole UK cohort. Using this methodology, I have reduced some of the error introduced to population-based studies, caused by admixture (Abi-Rached et al., 2011; Skotte et al., 2013). The AIMs panel used was concise and only allowed differentiation between European and non-European populations. There are other, more detailed ancestry panels which would have allowed more elaborate analysis and enabled us to create a geographically smaller subgroup (such as the United Kingdom or Northern Europe). There would have been benefits to this; a European subgroup includes individuals from Southern Europe and the Mediterranean – these populations share much of their genome with individuals from the Middle East and Northern Africa. It could be argued that including Italians in the same group as British individuals makes less sense than grouping Italians with Turkish individuals. This argument has merit. However, the smaller the geographic region analysed, the larger the overall dataset would need to be. In this project, I was limited to patients from the UK and therefore the pool of potential patients was relatively small. For this reason, the UK cohort has been treated as a single group throughout this thesis with reference and confirmation to the European subgroup where relevant. From a clinical perspective, it is not useful to be able to sub-categorise risk

factors that are applicable to individuals of one small geographic region as this kind of information is not readily available in a clinical setting.

Prior to investigating the effects of the *KIR3DL1/S1* 'functional genotypes', NK and CD8 T cell subsets were examined for presence of KIR3DL1/S1 in the BD group. Peripheral blood mononuclear cells were collected from 22 individuals with known *KIR3DL1/S1* alleles. I confirmed low percentages of circulating NK cells in our BD cohort, in line with previous data from our UK cohort (Hasan et al., 2017). I was unable to demonstrate any differences between active and inactive disease, or in those on disease-modifying therapy, although the sample size was small and meaningful differences cannot be excluded on the basis of this data.

Overall, KIR3DL1 was expressed on 5.22 ± 3.02% of NK cells, with the majority of individuals expressing less than 5% KIR3DL1. KIR3DL1 was present on the surface of CD8 T cells in 22.72% (n=5) of individuals tested and only at very low levels.

KIR3DS1 was possessed by 22.72% (n=5) of individuals tested and expressed on 1-5% of circulating NK cells. This is in contrast to work by Li et al., who suggested that in individuals with one copy of *KIR3DS1* 20-50% of NK cells express KIR3DS1, whereas in individuals with two copies, more than 60% of NK cells express the receptor (Li et al., 2008).

The target cells used for CD107a assays were 721.221 B lymphoblastoid cells, which were thought to be HLA-F null, however in 2018, Kiani et al recently demonstrated that 221-cells do indeed express HLA-F (Kiani et al., 2018). HLA-F OC are ligands for KIR3DS1 and therefore findings from assays involving KIR3DS1^{+} cells should be treated

with caution. However, in samples analysed, I found minimal expression of KIR3DS1 and its overall effect on CD107a expression is likely to be minimal.

Low expression of KIR3DL1/S1 is an important finding from this project, but also colours interpretation of CD107a assays subsequently carried out. I did not find any significant variation in CD107a expression in the individuals tested. However, this may reflect the relative paucity of KIR3DL1/S1 on the surface of cells tested. Much of the published data examining KIR3DL1-Bw4 interaction has been carried out using tetramer binding to characterise the binding patterns of KIR3DL1 to its ligand. This data may not be representative in BD. Low numbers of circulating NK cells could also be explained by the sequestration of KIR3DL1/S1-bearing NK cells in tissues, resulting in a depleted circulating population. Whether low expression of KIR3DL1/S1 is associated with disease pathogenesis or a secondary effect of depleted NK cells remains to be seen.

Finally, I sought to define the ophthalmic subset in greater detail by carrying out a prospective survey of ophthalmic BD in the UK using the BOSU reporting system. This allowed me to calculate incidence of ophthalmic disease in the UK over a period of 12 months as well as gather data regarding the presenting features of ophthalmic disease, the initial management strategy and referral pathways that exist for BD. The incidence of ophthalmic BD in the UK is 0.04 per 100,000 individuals, with male patients making up 52% of all cases reported. The median age at presentation was 37 years. Forty-eight percent of reported cases occurred in individuals with a previous diagnosis of BD and of these individuals, those concurrently treated with 2 or more DMARDS or a biologic agent had less severe ophthalmic disease that those taking corticosteroids alone or corticosteroids plus one DMARD. Patients with cystoid macula oedema presented with the worse vision than those with vitritis or retinal infiltrates (P=0.0102). Ophthalmic

manifestations of BD were treated with oral or topical corticosteroids most commonly, with intravenous and intravitreal corticosteroids being used in less than 10% of cases.

6.2 Limitations Of The Project

6.2.1 Clinical Data

It is possible that some of the clinical information collected was inaccurate. The clinical data was taken from the clinical records, however, some patients had missing notes and were unable to accurately recall the exact clinical manifestations of their disease since symptoms began. Furthermore, it is possible that some of the HCs were not truly 'healthy'. I relied on history from the unrelated HCs to ensure there was no current or past history of BD or any other inflammatory disease. However, patient recall is imperfect, and it is possible that some of these individuals had other diseases with *HLA, MICA* or *KIR3DL1/S1* associations that they were unaware of.

6.2.2 Genotyping

Despite our best efforts, some DNA was either too dilute for analysis, contaminated, or did not amplify during the library preparation. While there was *HLA-A* data available for over 95% of individuals and *HLA-B* data for 99%, a proportion of data was lost at the start. This may be relevant as some of the associations found were present in very low number of individuals. For example, the *HLA-B*58* association was present in less than 1% of BD cases.

In order to make the project affordable, samples were sequenced to a depth of 10-15x. This meant that some of the samples had ambiguous reads and had to be repeated using either SSP or SSOP depending on the quantity of DNA available. There were two cases from the HC group that were excluded as no DNA was available and the HC was not willing to donate more blood.

There are also intrinsic limitations to the data set used to derive the AIMs, which were taken from the Wellcome Trust Case Control Consortium 3 anorexia nervosa GWAS, which included 2,907 cases from 15 different populations of European origin. The samples were collected for clinical use rather than population genetics and details of the ancestry of these patients was not always available.

6.2.3 Statistical Analysis

This project was not carried out in conjunction with a statistical geneticist. Although I endeavoured where possible to ask opinions at international conferences and from of the groups working in the field, I carried out all the analyses myself. The project would have benefitted from early planning with a statistical geneticist and collaboration regarding the interpretation of results. As mentioned in the Chapter 2.12, I corrected multiple testing using Bonferroni-Dunn correction. This is well known to be a very stringent process and while it is likely to avoid type I error, it may lead to a number of relevant findings being dismissed.

Where relevant, I have calculated post-hoc power to give the results context with regard to type II errors. There are a large number of potential sources for type II error in the project and these have been highlighted throughout the thesis. Ultimately, a larger sample

size would have benefitted the analysis, however, BD is rare in the UK so larger numbers of samples were not an option for me.

6.2.4 Small Number Of Samples Available For Phenotypic And Functional Analysis

The low number of individuals with known KIR3DL1/S1 allotypes analysed is a major limitation of this project. It would have been preferable to store PBMC from all individuals recruited for the genotyping part of the project, thus a library of PBMC would have been available for later analysis. However, I did not have ethics approval to create a lymphocyte biobank and did not have the liquid nitrogen storage facilities to store 712 samples of PBMC. Furthermore, it is possible that some participants would have objected to donating more than 50 ml of blood at the time of venepuncture.

6.2.5 New Cases Of Ophthalmic Manifestations In The United Kingdom Are Rare Events

The British Ophthalmic Surveillance unit is set up to allow the incidence of rare diseases to be calculated. In this study, I received 23 reported of new cases over a 12-month period. With such small numbers a reporting error or a missed case can have a large impact on the overall incidence. In order to increase the confidence of this finding I have decided to run the study for a further 12 months.

6.3 Further Work

It would be of great use to carry out a large KIR3DL1/S1-phenotyping project in BD. Findings from this project have suggested that KIR3DL1/S1 expression is low in patients with BD. Future projects would benefit from data from HCs to compare against BD KIR3DL1/S1 expression.

This project did not examine *KIR3DL1* mRNA transcript levels in our cohort. However, given our findings, it would be informative to establish whether low expression of KIR3DL1 is due to an upstream transcription abnormality. Previous work investigating *KIR3DL1* mRNA transcripts in healthy Han Chinese individuals found no difference between different *KIR3DL1* alleles (Tao et al., 2014). Nonetheless, there has been some work carried out investigating KIR transcript levels in HIV-1 infection suggesting that increased copy number variation in *KIR3DL1,* in conjunction with the presence of *KIR3DS1* may lead to resistance to HIV-1 infection (Pelak et al., 2011).

As with any new genetic finding, the 'functional genotypes' elucidated here need to be explored in other BD cohorts around the world.

6.4 Summary Of Thesis Findings

This thesis focuses on genetic risk factors and ophthalmic manifestations in a cohort of individuals with BD from the UK. There is an established association between BD and risk loci within *HLA*. I analysed *HLA-A* and *-B* as well as *MICA* in cohort of 267 BD patients and 445 HC. I confirmed *HLA-B*51* (OR 2.13 (1.49-3.06), *Pc*=0.0009) and *MICA*009* (OR 1.80 (1.31-2.47), *Pc*=0.0045) as independent risk factors for BD in a

mixed UK cohort and a subgroup of individuals with European ancestry-informative markers (with a smaller effect size). I then investigated the associations between *KIR3DL1/S1* and BD. *KIR3DL1/S1* allele-level analysis indicated that low-expressing *KIR3DL1/S1* alleles in combination with *KIR3DS1* increased the risk of developing BD (*KIR3DL1*^{LOW}*/KIR3DS1: P*=0.0004, *Pc*=0.0040, OR 2.47, 95% CI 1.43-4.25), whereas high-expressing *KIR3DL1/S1* alleles in combination with a null-expressing *KIR3DL1* reduced the risk of disease (*KIR3DL1*^{HIGH}*/KIR3DL1*^{NULL}: *P*=0.0035, *Pc*=0.0350, OR 0.53, 95% CI 0.33-0.87). Behçet's Disease can manifest as a purely mucocutaneous disease or can involve other organ systems such as the eyes. In the UK cohort studied here, *KIR3DL1*^{LOW}*/KIR3DS1* increased the risk of ophthalmic disease (*P*=1.2x10^{-5}, OR 3.92, 95% CI 2.06-7.47), whereas *KIR3DL1*^{HIGH}*/KIR3DL1*^{NULL} provided protection against mucocutaneous disease (*P*=0.0048, OR 0.45, 95% CI 0.25-0.81). I then investigated expression of KIR3DL1/S1 in a genotyped group of individuals, but found only very low levels expression (1-5%) in the majority of cases; limiting further functional work. Finally, I undertook a prospective study to analyse ophthalmic manifestations BD in the UK and found the incidence to be 0.04 (95% CI 0.02-0.05) per 100,000. This is the first piece of work to explore both the genetic risk factors in a UK cohort of individuals with BD and the incidence of eye-disease in this group of patients.

7 Publications

1. The combination of a low expressing KIR3DL1 allotype and KIR3DS1 contributes toward the risk of developing Behçet's Disease in the United Kingdom. **Petrushkin H**, Norman PJ, Lougee E, Parham P, Wallace GW, Stanford MR, Fortune F *Submitted to* J Immunology 12/9/2018

2. The Use of Topical Non-steroidal Anti-inflammatory Drugs for Uveitic Cystoid Macular Edema. **Petrushkin H**, Rogers D, Pavesio C. Ocular Immunology and Inflammation. 2017 Jan 12:1-3.

3. Case-control study of risk factors for acute corneal hydrops in keratoconus. Barsam A, Brennan N, **Petrushkin H**, Xing W, Quartilho A, Bunce C, Foot B, Cartwright NK, Haridas A, Agrawal P, Suleman H, Ahmad S, MacDonald E, Johnston J, Tuft S. British Journal of Ophthalmology. 2016 Jul 7.

4. Improving morbidity and mortality in peripheral ulcerative keratitis associated with rheumatoid arthritis. **Petrushkin H**, Stanford M, Fortune F, Jawad A. *Clinical and experimental rheumatology*. 2016 Jan-Feb;34(1 Suppl 95):S18-9.

5. Intermediate uveitis associated with familial Mediterranean fever. **Petrushkin H**, Karagiannis D, Bird A, Jawad A. *Clinical and experimental rheumatology*. 2015 Nov-Dec;33(6 Suppl 94):S170

6. Gamma Delta (γδ) T Cells and Their Involvement in Behçet's Disease. Hasan MS, Bergmeier L, **Petrushkin H**, Fortune F. *Journal of Immunology Research* vol. 2015, Article ID 705831, 7 pages, 2015

7. Intermediate uveitis and multiple sclerosis: To scan or not to scan. **Petrushkin H**, Kidd D, Pavesio C. The British journal of ophthalmology 09/2015; 99(12).

8. Acute corneal hydrops in keratoconus: a national prospective study of incidence, epidemiological factors and treatment strategies. Barsam A, **Petrushkin H**, Brennan N, Bunce C, Xing W, Foot B, Tuft S. Eye. 29, 2015 469–474

9. Behçet's Disease: Do Natural killer Cells Play a Significant Role? **Petrushkin H**, Wallace G, Stanford M, Fortune F. Frontiers In Immunology. 2015 Mar 24;6:134.

10. Clinical Review: Familial Mediterranean Fever- An Overview of Pathogenesis, Symptoms, Ocular Manifestations, and Treatment. **Petrushkin H**, Stanford M, Jawad A. Ocular Immunology and Inflammation. 2016 Aug;24(4):422-30.

11. Does possession of the *HLA-DRB1*1501* allele influence visual outcome in idiopathic intermediate uveitis? **Petrushkin H**, Thomas D, Vaughan R, Kodeatis E, Stanford M, Edelsten C, Wallace G. JAMA Ophthalmology. 2015;133(4):482-3

12. Continuing Progress in Vasculitis Research. Abstracts of the 17th International Vasculitis & ANCA Workshop. **Petrushkin H**, Fortune F. *Nephron*. 2015;129 Suppl 2:1-44

13. Rescue Pneumatic Retinopexy for Recurrent Retinal Detachment. **Petrushkin H**, Elgohary M, Sullivan P. Retina. 2015 Sep;35(9):1851-9.

14. Intralesional Steroid for Orbital Manifestations of Rosai-Dorfman Disease. **Petrushkin H**, Salisbury J, O'Sullivan E. Clinical and Experimental Ophthalmology. 2015 Jul;43(5):483-5.

15. The Eye - Oxford Textbook of Rheumatology, 4th Edition. **Petrushkin H**, Stanford M. 2013-16. Oxford University Press

8 Conference Presentations

KIR3DL/S1 functional genotypes confer risk in Behçet's Disease. International Behçet's Disease Conference, Rotterdam, The Netherlands, 2018. Petrushkin H, Norman P, Lougee E, Stanford M, Parham P, Fortune F. Awarded Young Investigator of the Year

KIR3DL/S1 functional genotypes confer risk in Behçet's Disease. KIR Workshop, Camogli, Italy 2018. Petrushkin H, Norman P, Lougee E, Stanford M, Parham P, Fortune F.

Comorbidities in Behçet's Disease, International Behçet's Disease Conference, Matera, Italy 2016. Petrushkin H, Hasan S, Senusi A, Fortune F.

9 Division Of Labour

The following individuals were involved in this project. Affiliations are in superscript following each individual's initials.

Harry Petrushkin, HP[1]. Farida Fortune, FF[1]. Angray Kang, AK[1]. Meleri Jones, MJ[1]. Gary Warnes, GW[1]. Amal Senusi, AS[1]. M. Samiul Hasan, SH[1]. Jie Liu, JL[1]. Miles Stanford, MS[2]. Graham Wallace, GW[3]. Charles Mein, CM[4]. Ian Donaldson, ID[4]. Anna Terry, AT[4]. Paul Norman, PN[5]. Barny Foot, BF[6]. Emma Lougee, EL[7]. Sahar Parvizi, SP[8]. Nardos Wakjira, NW[9]. Melanie Day, MD[9]. Elizabeth Ssendi, ES[9].

[1]Clinical and Diagnostic Oral Sciences, Queen Mary University London, Blizard Institute, 4 Newark St, E1 2AT, London, UK

[2]Medical Eye Unit, St Thomas' Hospital, Westminster Bridge Rd, SE1 7EH, London UK

[3]Centre for Translational Inflammation Research, University of Birmingham Research Laboratories, Queen Elizabeth Hospital, University of Birmingham, Edgbaston, B15 2TT, Birmingham, UK

[4]Genome Centre, Barts and the London School of Medicine and Dentistry, John Vane Science Centre, Charterhouse Square, EC1M 6BQ, London, UK

[5]Parham Laboratory, Department of Structural Biology, Stanford University School of Medicine, D159, Sherman Fairchild Science Building, 299 Campus Drive West, Stanford, CA 94305, United States of America

[6]British Ophthalmic Surveillance Unit, Royal College of Ophthalmologists, 18 Stephenson Way, Kings Cross, NW1 2HD, London, UK

[7] Viapath, Clinical Transplantation Laboratory, 3rd Floor Borough Wing, Guy's Hospital, Guy's and St Thomas' NHS Foundation Trust, Great Maze Pond, SE1 9RT, London, UK

[8]Moorfields Eye Hospital, 162 City Rd, EC1V 2PD, London, UK

[9]Behçet's Centre of Excellence, Royal London Hospital, Bart's and the London NHS Foundation Trust, Whitechapel Rd, E1 1BB, London, UK

Chapter 1	Literature Review and Discussion	HP
Chapter 2	Methodology	HP
Chapter 3	Design of NGS panel	CM, HP, GW, MS, FF
	Venepuncture	MD, HP, NW, ES, AS, JL
	Informatics	AT, ID
	AIMs analysis	ID
	Data Analysis	HP
	HLA-A and B typing	HP, EL
	Interpretation of results	HP, PN, FF, GW, MS, FF
Chapter 4	Design of NGS panel	CM, HP, GW, MS, FF
	Informatics	AT, ID
	AIMs analysis	ID
	Data Analysis	HP
	Interpretation of results	HP, PN, FF, GW, MS, FF
	Design / construction of vectors	HP, AK
	Transfection of cell lines	HP, MJ
	Degranulation assays	HP
	Flow cytometry	HP, GW
Chapter 5	Design of questionnaire	HP, SP, MS, BF
	Collection of data	HP, SP
	Data analysis	HP
	Interpretation of results	HP, GW, MS, FF

10 Acknowledgements

Thank you to all of my colleagues and collaborators mentioned above for your help with different aspects of the work described in this thesis. I am grateful to all my colleagues in Clinical and Diagnostic Oral Sciences for their support and advice over the past four years. I am indebted to Angray Kang, for taking the time to teach me the basics of molecular biology, without whom I would not have been able to construct the cell lines used in this project.

I would like to thank Robert Vaughan, Elli Konteadis and Emma Lougee in the clinical transplantation laboratory at Guy's Hospital for sharing their expertise over the past eight years and supervising all the SSP and SSOP typing that was used in this project.

Olympe Chazara, at the Centre for Trophoblast Research arranged the donation of cell lines from Ashley Moffatt's laboratory and offered invaluable advice at the start of the project. I feel very lucky to have been introduced to the KIR-community and have benefitted from many discussions with Paul Norman, Steve Marsh, Becca Asquith and Peter Parham over the past four years.

This work would not have been possible without the support from the clinical team at the Behçet's Centre of Excellence and I would like to thank everyone responsible for consenting patients, taking blood, extracting PBMC and building our database of patients. In particular, I would like to acknowledge Leslie Bergmeier for teaching me laboratory techniques and assisting with troubleshooting when I started in the laboratory. I would also like to thank all the patients that attend the clinic who donated samples as well as all the healthy controls.

To my funders: The Frost Charitable Trust, the Royal College of Ophthalmology and Bart's Charity - thank you for supporting me financially through my PhD.

To my supervisors: Miles Stanford's inspirational teaching sessions were the reason I became an ophthalmologist, then went into uveitis and then discovered Behçet's Disease. Graham Wallace conceived this project and has always been available to offer me advice and support. Farida Fortune, has been a mentor, a teacher and one of the most caring clinicians I have had the chance to work with. Without Prof Fortune, this project would have had no home, no patients and no funding - I am so grateful. Thank you all.

Finally: Natalie, Arlo and Poppy, thanks for an amazing four years, it would have been no fun without you.

11 Appendix 1 – Primers For PCR SSP *HLA-A* And *HLA-B*

Table 11-1. PCR SSP Primers Used for HLA-A and –B Typing

Forward Primer Primer No.	HLA	Annealing Position	
174	HLA-A	241-259	5' C C G G A G T A T T G G G A C C T G C
187	HLA-B	217-234	5' G C G C C G T G G A T A G A G C A A
188	HLA-B	190-206	5' G C C G C G A G T C C G A G G A C
189	HLA-B	254-272	5' A C C G G A A C A C A C A G A T C T G
192	HLA-B	254-272	5' A C C G G G A G A A C A C A G A T C T C
193	HLA-A&B	243-261	5' G G A G T A T T G G G A C C G G A A C
194	HLA-B	268-285	5' A A C A T G A A G G C C T C C G C G
195	HLA-B	253-272	5' G A C C G G A A C A C A C A G A T C T T
197	HLA-B	230-246	5' A G C A G G A G G G G C C G G A A
202	HLA-B	124-141	5' G G G G A G C C C C G C C T T C A T T
203	HLA-B	265-283	5' C A G A T C T A C A A G G C C C A G G
205	HLA-B	83-103	5' C C A T G A G G T A T T C T A C A C C G
206	HLA-B	253-272	5' G A C C G G A A C A C A C A G A T C T A
207	HLA-B	294-311	5' C C G A G A G A G C C T G C G G A A
208	HLA-A&B	293-311	5' A C C G A G A G A A C C T G C G G A T
209	HLA-B	189-206	5' C G C C G C G A G T C C G A G A G A
239	HLA-A	406-423	5' G G G T A C C A G C A G G A C G C T
240	HLA-B	259-278	5' G A G A C A C A G A A A G T A C A A G C G
242	HLA-B	295-312	5' C G A G A G A G C C T G C G G A A C
243	HLA-B	192-209	5' C G C G A G T C C G A G G A T G G C
246	HLA-B	78-97	5' C C A C T C C A T G A G G T A T T T C C
271	HLA-B	125-142	5' G G G A G C C C C G C T T C A T C T
272	HLA-B	189-206	5' C G C C A C G A G T C C G A G G A A
280	HLA-B	149-167	5' G C T A C G T G G A C G A C A C G C T
284	HLA-A	247-265	5' T A T T G G G A C G A G G A G A C A G
286	HLA-A	186-203	5' C G A C G C C G C G A G C C A G A A
288	HLA-A	283-302	5' T C A C A G A C T G A C C G A G C G A A
290	HLA-A	264-282	5' A C G G A A A T G T G A A A G G C C C A G
291	HLA-A	184-200	5' A G C G A C G C C G C G A G C C A
292	HLA-A	239-257	5' G G C C G G A G T A T T G G G A C G A
294	HLA-A&B	283-302	5' T C A C A G A C T G A C C G A G A G A G
295	HLA-A	110-126	5' C C C G G C C C G G C A G T G G A
296	HLA-A	222-240	5' G T G G A T A G A G C A G G A G G G T
368	HLA-B	284-302	5' C A C A G A C T G A C C G A G T G A G
371	HLA-A	78-98	5' C C A C T C C A T G A G G T A T T T C T C
434	HLA-A	78-98	5' C C A C T C C A T G A G G T A T T T C A C
435	HLA-B	303-319	5' C C T G C G C A C C G C G C T C C
451	HLA-A	406-423	5' G G G T A C C G G C A G G A C G C T
475	HLA-A	264-282	5' A C G G A A A G T G A A A G G C C C A G
493	HLA-A	161-180	5' C A C G C A G T T C G T G C G G T T T
395	HLA-B	190-206	5' G C C G C G A G T *T* C G A G A G G

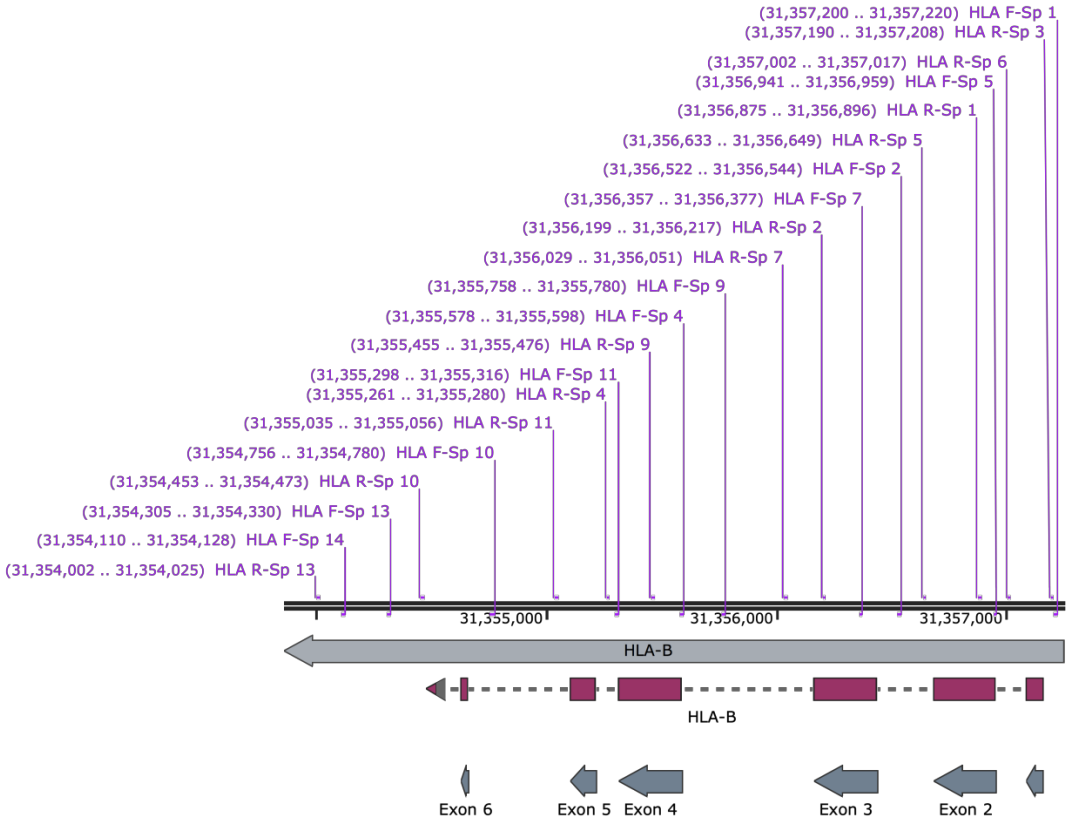
Reverse Primer Primer No.	HLA	Annealing Position	
127	HLA-B	361-379	5' G G T C G C A G C C A T A C A T C C A
145	HLA-A&B	559-576	5' G A G C C A C T C C A C G C A C T C
146	HLA-A	539-557	5' C C C T C C A G G T A G G C T C T C T
167	HLA-A	559-576	5' G A G C C A C T C C A C G C A C C G
168	HLA-A&B	559-576	5' G A G C C A C T C C A C G C A C G T
170	HLA-A	538-556	5' C C T C C A G G T A G G C T C T C T G
171	HLA-A	391-407	5' C C G C G G A G G A A G C G C C A
184	HLA-B	512-528	5' C G C A C G G G C C G C C T C C A
213	HLA-B	387-402	5' G A G G A G G C G C C C G T C G
214	HLA-A&B	419-435	5' C T T G C C G T C G T A G G C G G
215	HLA-B	420-438	5' A T C C T T G C C G T C G T A G G C T
216	HLA-B	435-454	5' C G T T C A G G G C G A T G T A A T C T
217	HLA-B	544-561	5' C G T G C C C T C C A G G T A G G T
218	HLA-A&B	559-576	5' G A G C C A C T C C A C G C A C T C
219	HLA-B	572-589	5' C C A G G T A T C T G C G G A G C G
220	HLA-B	603-619	5' C C G C G C G C T C C A G C G T G
221	HLA-B	605-622	5' T A C C A G C G C G C T C C A G C T
223	HLA-B	353-372	5' G C C A T A C A T C C T C T G G A T G A
224	HLA-B	361-379	5' C G T C G C A G C C A T A C A T C A C
225	HLA-B	527-544	5' C T C T C A G C T G C T C C G C C T
228	HLA-A&B	411-438	5' T C G T A G G C G T C C T G G T G G
229	HLA-B	499-516	5' C T C C A A C T T G C G C T G G G A
232	HLA-B	246-265	5' G T G T G T T C C G G T C C C A A T A T
236	HLA-B	354-371	5' C C A T A C A T C G T C T G C C A A
237	HLA-B	302-318	5' G C G C A G G T T C C G C A G G C
238	HLA-A&B	559-576	5' G A G C C A C T C C A C G C A C A G
241	HLA-B	463-479	5' G C C G C G G T C C A G G A G C T
244	HLA-A&B	571-588	5' C A G G T A T C T G C G G A G C C C
247	HLA-B	463-479	5' G C G G C G G T C C A G G A G C G
249	HLA-A&B	538-556	5' C C T C C A G G T A G G C T C T C A A
250	HLA-B	299-316	5' G C A G G T T C C G C A G G C T C T
276	HLA-A&B	499-515	5' T C C C A C T T G C G C T G G G T
277	HLA-B	387-403	5' G G A G G A A G C G C C G T C G
281	HLA-B	280-298	5' C T C G G T C A G T C T G T G C C T T
282	HLA-B	280-299	5' T C T C G G T A A G T C T G T G C C T T
285	HLA-B	412-430	5' C G T C G T A G G C G T A C T G G T C
287	HLA-A&B	559-576	5' G A G C C C G T C C A C G C A C T C
298	HLA-A	423-443	5' A T G T A A T C C T T G C C G T C G T A A
299	HLA-A	555-572	5' C A C T C C A C G C A C G T G C C A
300	HLA-A	448-466	5' A G C G C A G G T C C T C G T T C A A
301	HLA-A	414-431	5' C C G T C G T A G G C G T G C T G T
302	HLA-A	453-471	5' C C A A G A G C G C A G G T C C T C T
303	HLA-A	527-544	5' C T C T C T G C T G C T C C G C C G
377	HLA-B	538-556	5' C C T C C A G G T A G G C T C T C C A
392	HLA-B	419-436	5' C C T T G C C G T C G T A G G C G A
393	HLA-B	412-429	5' G T C G T A G G C G T C C T G G T C
394	HLA-A	364-382	5' C C A C G T C G C A G C C A T A C A T T
429	HLA-A	259-278	5' G C C T T C A C A T T C C G T G T G T T
431	HLA-A	559-575	5' A G C C C G T C C A C G C A C C G
486	HLA-A	257-276	5' C T T C A C A T T C C G T G T C T C C T
494	HLA-A	299-316	5' G C A G G G T C C C C A G G T C C A

12 Appendix 2 – *HLA- B* Sequencing Primers

Table 12-1. HLA-B Primers Used for Library Preparation

Name	Primer
HLA F-Sp 1	CACGTAGCCCACTGAGATGAAG
HLA F-Sp 2	AGGTATCTGCGGAGCCACT
HLA F-Sp 3	GGGTGCGTGGGGACTTTAG
HLA F-Sp 4	CTGAAGGGCTCCTGCTTTCC
HLA F-Sp 5	GCCGTACGTGGGGGATG
HLA F-Sp 6	CGCGGCTCCTCAGGTC
HLA F-Sp 7	ACTCAGGAAAACTCATGCCATTC
HLA F-Sp 8	CGACACAAGTTGGGAGAAGAAGT
HLA F-Sp 9	CAGTGTCCTGAGTTTGGTCCTC
HLA F-Sp 10	CCCACTCTAGACCCCAAGAAT
HLA F-Sp 11	CACTTCTACCTGGGGCTTGAAA
HLA F-Sp 12	GTCCTGATCCCTCTTCTCCTACA
HLA F-Sp 13	GGAAGTAAGAAGTTGCAGCTCAGT
HLA F-Sp 14	CCCCCACAGCCTTCTCCA
HLA R-Sp 1	GCGGTCCCAGTTCTAAAGTCC
HLA R-Sp 2	GCGTTTACCCGGTTTCATTTTCA
HLA R-Sp 3	TGTAGGAGAAGAGGGATCAGGAC
HLA R-Sp 4	CATCAGACCCCCCAAAGACAC
HLA R-Sp 5	AGGCTCCCACTCCATGAGG
HLA R-Sp 6	ACTTCTTCTCCCAACTTGTGTCG
HLA R-Sp 7	CATGACCAGTACGCCTACGAC
HLA R-Sp 8	GTGCTCAGTTTCCCTACACAAGA
HLA R-Sp 9	GCTCAGAGACTCGAACTTTCCAA
HLA R-Sp 10	GGGTCTGTAGTCATACTTCTGGAAA
HLA R-Sp 11	GTAAGGAGGGGGATGAGGG
HLA R-Sp 12	GCGGTGCCTTCAGAGAAAACT
HLA R-Sp 13	ACCAGAATTTGTTCATGACTGTTGTT
HLA R-Sp 14	GTGACCCCTGTTCCCATGC

Figure 12-1. Map of HLA-B Amplicons

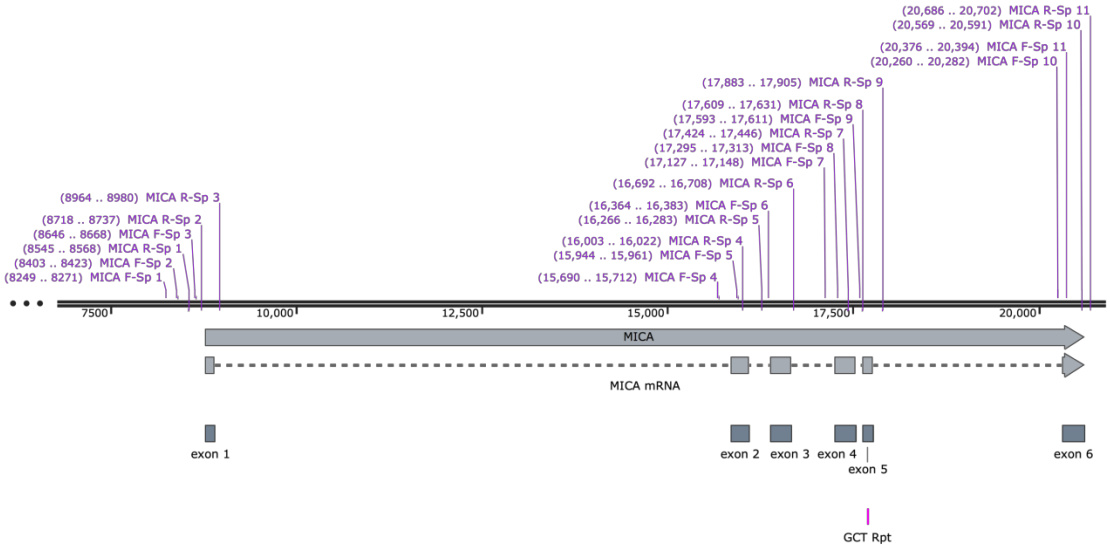


13 Appendix 3 – MICA Sequencing Primers

Table 13-1. MICA Primers Used for Library Preparation

Name	Primer
MICA F-Sp 1	GTTTGTGGTCTGAGCGGTTCTAC
MICA F-Sp 2	TCTGTCCTGGAAGGAACAAGC
MICA F-Sp 3	TTCTAAATCTCCCCAGGTCTCCA
MICA F-Sp 4	TATCCTCCCACCCTCACAGTTTT
MICA F-Sp 5	GATGGTCAGCCCTTCCTG
MICA F-Sp 6	GAGAAGTCACTGCTGGGTGG
MICA F-Sp 7	CAGAGTGAGGACAGACTTGCAG
MICA F-Sp 8	AGGCCTCAGAGGGCAACAT
MICA F-Sp 9	GGGATGGCTGTGGCTCTCT
MICA F-Sp 10	AGTCCCCAGGGAATAAACACAAC
MICA F-Sp 11	CCACAGGGATGCCACACAG
MICA R-Sp 1	AGTACAGCTCCAACTTCATAAGCA
MICA R-Sp 2	CCCGGAACTTAGGGCCAATC
MICA R-Sp 3	CGACCCCCGGAAACGTC
MICA R-Sp 4	CCAGGACATCTTCTGCCCAC
MICA R-Sp 5	CCCTGTGTGGGCTGAGTG
MICA R-Sp 6	GGAGAGGAGAGCCCCTG
MICA R-Sp 7	CTGGTAGGTTCCATTCCCATCAG
MICA R-Sp 8	GGACTTGTTATACACTGGGCAGA
MICA R-Sp 9	AATTCCCCAACTTTCATCCCCTG
MICA R-Sp 10	ATTTTGCAGCCTCCAACAACAAT
MICA R-Sp 11	CCGTGCCTGGCCTGAGA

Figure 13-1. Map of HLA-B Amplicons

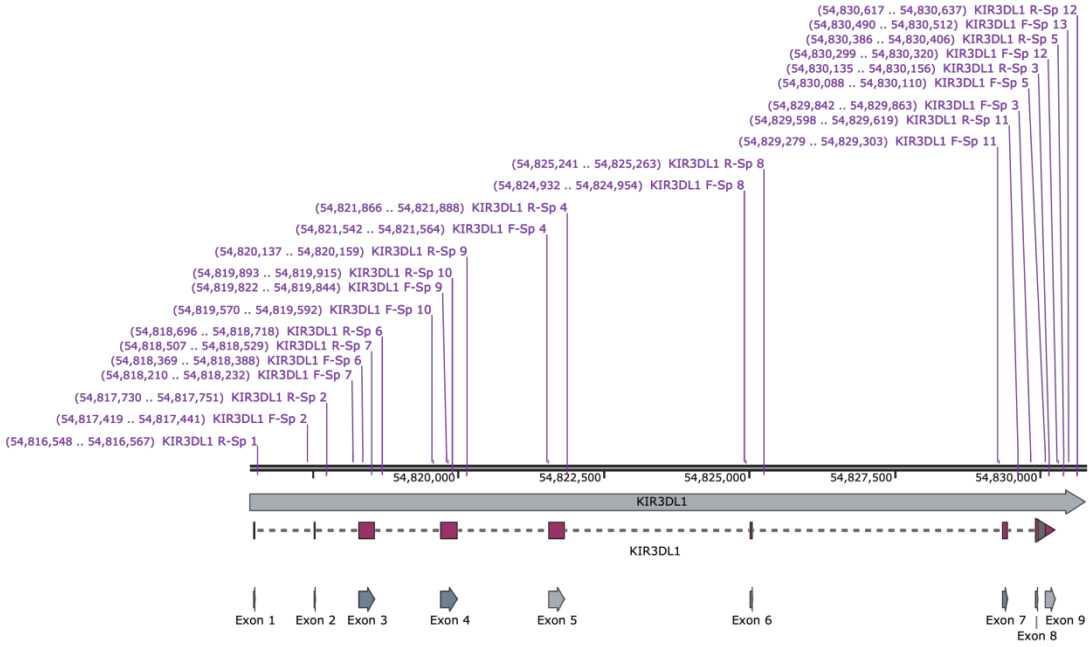


14 Appendix 4 – KIR3DL1 Sequencing Primers

Table 14-1. KIR3DL1 Primers used for Library Preparation

Name	Primer
KIR3DL1 F-Sp 1	AGACGTGTTTTGAGTTGGTCATAGT
KIR3DL1 F-Sp 2	GAGTTTACCTTCAGCCCAGCAAG
KIR3DL1 F-Sp 3	CCTCCCCCTGTTTGTTGGTATC
KIR3DL1 F-Sp 4	GAAACTGCCTCTTCTCCTTCCAG
KIR3DL1 F-Sp 5	CCTCTCTCCAGGACTCTGATGAA
KIR3DL1 F-Sp 6	GAGGACACGTGACTCTTCGG
KIR3DL1 F-Sp 7	TGGTAGGAGCCTTAGAAAGTGGA
KIR3DL1 F-Sp 8	TAACAGAGTGTTGGCCATGAACC
KIR3DL1 F-Sp 9	GCACAAAGAGGGGATCTCTAAGG
KIR3DL1 F-Sp 10	GGGATATGGGCACAGAAAAGACA
KIR3DL1 F-Sp 11	CCTGTCAATCAAGAAATGCAAGACA
KIR3DL1 F-Sp 12	CGTCTTCTAGGGAGACAACAGC
KIR3DL1 F-Sp 13	GCTTAGCCCACAGTTCTCCATTT
KIR3DL1 F-Sp 14	GAGACAGAGAGAGAGCAAACCATGAG
KIR3DL1 F-Sp 15	TGCATAATCCTTCCCTGGGAGTT
KIR3DL1 R-Sp 1	CACTCCCTCCCTCGATTCCC
KIR3DL1 R-Sp 2	CAGAGCTTGACTCAGGAAAGGG
KIR3DL1 R-Sp 3	AAACGCAGTGATCCAACTGTGC
KIR3DL1 R-Sp 4	GGACATGAGAGAGATATGGGCTT
KIR3DL1 R-Sp 5	GAGGAGCGATGCCCTAAGATG
KIR3DL1 R-Sp 6	CTCCCTTGACCCCAAATACAGTC
KIR3DL1 R-Sp 7	ACATGTGTAGTTCCCTGCATGTG
KIR3DL1 R-Sp 8	TAAGAGACTTCGCTGAGCCCTTT
KIR3DL1 R-Sp 9	GTCTCTGTTGGTACAGACCTCAC
KIR3DL1 R-Sp 10	CATCATGGGACCGATGGAGAAAT
KIR3DL1 R-Sp 11	AGGGAGTCTGGTGCTCTCTCTA
KIR3DL1 R-Sp 12	GTAAGTGCCACGTCAAGAGGG
KIR3DL1 R-Sp 13	AGCGGTTTCTTTCAGCGAATACA
KIR3DL1 R-Sp 14	TTTTATCTGAGATTCAAACTCTTCTTCCTGTG
KIR3DL1 R-Sp 15	CAGTGGGTGCTCGCTCA

Figure 14-1. Map of KIR3DL1 Amplicons



15 Appendix 5 - Questionnaire for Reporting Clinicians – Prospective Surveillance of Ophthalmic Manifestations of Behçet's Disease.

351436

Study code OBD/87

Ophthalmic manifestations of Behçet's Disease; a national prospective study of incidence and management.

In cooperation with the British Ophthalmological Surveillance Unit (BOSU) - A national population based study to determine the incidence and treatment strategies in the United Kingdom.

Age ___

Gender ❑ Male ❑ Female

First half of postcode _____

Ethnicity

White	❑ British ❑ Irish Other White (Please specify) _____
Mixed/multiple ethnic groups	❑ White and Black Caribbean ❑ White and Asian ❑ White and Black African Other Mixed (Please specify) _____
Asian/Asian British	❑ Indian ❑ Pakistani ❑ Bangladeshi ❑ Chinese Other Asian (Please specify)_____
Black/ African/ Caribbean/ Black British	❑ African ❑ Caribbean Other Black (Please specify) _____
Other Ethnic groups	❑ Middle Eastern Any other ethnic group (Please specify)_____

Which eye(s) are affected ❑ Right ❑ Left ❑ Both

Date patient first seen with ophthalmic manifestation of Behçet's Disease DD/MM/YYYY

Date Behçet's Disease was diagnosed (if applicable) MM/YYYY

Year of first systemic symptom of BD YYYY

Mr Harry Petrushkin - Uveitis Fellow, Medical Retina Department, Moorfields Eye Hospital
Telephone: 0207 566 2036 E-mail: harry.petrushkin@moorfields.nhs.uk

351436

Study code OBD/87

Systemic manifestations of Behçet's Disease at any point in the past

❑ **None**

❑ **Recurrent Oral Ulceration:**

Minor aphthous, major aphthous, or herpetiform ulceration observed by physician or patient, which recurred at least 3 times in one 12-month period.

❑ **Recurrent genital ulceration:**

Aphthous ulceration or scarring, observed by a physician or patient.

❑ **Eye lesions:**

Anterior uveitis, posterior uveitis, or cells in vitreous on slit lamp examination; or retinal vasculitis observed by an ophthalmologist.

❑ **Skin lesions:**

Erythema nodosum observed by physician or patient, pseudofolliculitis, or papulopustular lesions; or Acneiform nodules observed by a physician in post-adolescent patients not on corticosteroids.

❑ **Positive Pathergy test**

Read by a physician at 24 - 48 hours

Other _____

Best Corrected Visual Acuity at presentation **RE: _____** **LE:_____**

Reason for poor visual acuity (if reduced)

	Right Eye	Left Eye
Amblyopia	❑	❑
Corneal Opacity	❑	❑
Cataract	❑	❑
Vitritis	❑	❑
Macula Oedema	❑	❑
Ischaemia	❑	❑
Other		

Mr Harry Petrushkin - Uveitis Fellow, Medical Retina Department, Moorfields Eye Hospital
Telephone: 0207 566 2036 E-mail: harry.petrushkin@moorfields.nhs.uk

351436
Study code OBD/87

Ocular Manifestations of Behcet's Disease

	Right Eye	Left Eye
Ocular Surface Inflammation	Specify_____	Specify_____
Scleritis/ Episcleritis	Specify_____	Specify_____
Anterior Uveitis	❑	❑
Hypopyon	❑	❑
Posterior Synechiae	❑	❑
Vitritis	❑	❑
Macula Oedema	❑	❑
Retinal Infiltrate (see case definition)	❑	❑
Vascular occlusion (central, branch, multiple, peripheral)	Specify_____	Specify_____
Neovascularisation	Specify_____	Specify_____
Other		

Existing Treatment prior to onset of eye disease

Oral Steroid	❑
Conventional Immunosuppressive	❑
Multiple Immunosuppressives	❑
Biologic	❑
Steroid Mouth Wash	❑

First line treatment for ophthalmic manifestation of Behcet's Disease

	Right Eye	Left Eye
Topical steroid	❑	❑
Topical intraocular pressure-lowering agent	❑	❑
Intravitreal steroid	❑	❑
Periorbital steroid	❑	❑
Oral steroid	❑	❑
Conventional Immunosuppression	❑	❑
Intravenous Methylprednisolone	❑	❑

Mr Harry Petrushkin - Uveitis Fellow, Medical Retina Department, Moorfields Eye Hospital
Telephone: 0207 566 2036 E-mail: harry.petrushkin@moorfields.nhs.uk

351436
Study code OBD/87

Was the patient referred **from** another centre? Yes ❑ No ❑
Please Specify_____
(please note this information is for validation purposes only)

Was the patient referred **to** another centre for further treatment? Yes ❑ No ❑
Please Specify_____
(please note this information is for validation purposes only)

Form completed by _____

Thank you for taking the time to fill in this questionnaire

Please return the questionnaire to in the envelope provided to:

Harry Petrushkin - Uveitis Fellow
Medical Retina Department
Moorfields Eye Hospital
EC1V 2PD
London

16 References

Abi-Rached, L., Jobin, M. J., Kulkarni, S., McWhinnie, A., Dalva, K., Gragert, L., . . . Parham, P. (2011). The shaping of modern human immune systems by multiregional admixture with archaic humans. *Science, 334*(6052), 89-94. doi:10.1126/science.1209202

Ahn, R. S., Moslehi, H., Martin, M. P., Abad-Santos, M., Bowcock, A. M., Carrington, M., & Liao, W. (2016). Inhibitory KIR3DL1 alleles are associated with psoriasis. *Br J Dermatol, 174*(2), 449-451. doi:10.1111/bjd.14081

Akdeniz, S., Harman, M., Atmaca, S., & Akpolat, N. (2003). The seroprevalence of varicella zoster antibodies in Behçet's and other skin diseases. *Eur J Epidemiol, 18*(1), 91-93.

al-Dalaan, A. N., al Balaa, S. R., el Ramahi, K., al-Kawi, Z., Bohlega, S., Bahabri, S., & al Janadi, M. A. (1994). Behçet's disease in Saudi Arabia. *Journal of Rheumatology, 21*(4), 658-661.

Al-Okaily, F., Al-Rashidi, S., Al-Balawi, M., Mustafa, M., Arfin, M., & Al-Asmari, A. (2016). Genetic Association of HLA-A*26, -A*31, and -B*51 with Behçet's Disease in Saudi Patients. *Clin Med Insights Arthritis Musculoskelet Disord, 9*, 167-173. doi:10.4137/CMAMD.S39879

Al-Rawi, Z. S., Sharquie, K. E., Khalifa, S. J., Al-Hadithi, F. M., & Munir, J. J. (1986). Behçet's disease in Iraqi patients. *Ann Rheum Dis, 45*(12), 987-990.

Altenburg, A., Papoutsis, N., Orawa, H., Martus, P., Krause, L., & Zouboulis, C. C. (2006). [Epidemiology and clinical manifestations of Adamantiades-Behçet disease in Germany -- current pathogenetic concepts and therapeutic

possibilities]. *J Dtsch Dermatol Ges, 4*(1), 49-64; quiz 65-46. doi:10.1111/j.1610-0387.2006.05841.x

Ambresin, A., Tran, T., Spertini, F., & Herbort, C. (2002). Behçet's disease in Western Switzerland: epidemiology and analysis of ocular involvement. *Ocul Immunol Inflamm, 10*(1), 53-63.

Amroun, H., Djoudi, H., Busson, M., Allat, R., El Sherbini, S. M., Sloma, I., . . . Tamouza, R. (2005). Early-onset ankylosing spondylitis is associated with a functional MICA polymorphism. *Hum Immunol, 66*(10), 1057-1061. doi:10.1016/j.humimm.2005.09.004

Arnaiz-Villena, A., Karin, M., Bendikuze, N., Gomez-Casado, E., Moscoso, J., Silvera, C., . . . Martinez Laso, J. (2001). HLA alleles and haplotypes in the Turkish population: relatedness to Kurds, Armenians and other Mediterraneans. *Tissue Antigens, 57*(4), 308-317.

Azizlerli, G., Kose, A. A., Sarica, R., Gul, A., Tutkun, I. T., Kulac, M., . . . Disci, R. (2003). Prevalence of Behçet's disease in Istanbul, Turkey. *Int J Dermatol, 42*(10), 803-806.

Azuz-Lieberman, N., Markel, G., Mizrahi, S., Gazit, R., Hanna, J., Achdout, H., . . . Mandelboim, O. (2005). The involvement of NK cells in ankylosing spondylitis. *Int Immunol, 17*(7), 837-845. doi:10.1093/intimm/dxh270

Bahram, S. (2000). MIC genes: from genetics to biology. *Adv Immunol, 76*, 1-60.

Bahram, S., Bresnahan, M., Geraghty, D. E., & Spies, T. (1994). A second lineage of mammalian major histocompatibility complex class I genes. *Proc Natl Acad Sci U S A, 91*(14), 6259-6263.

Bancroft, G. J. (1993). The role of natural killer cells in innate resistance to infection. *Curr Opin Immunol, 5*(4), 503-510.

Bang, D., Choi, B., Kwon, H. J., Lee, E. S., Lee, S., & Sohn, S. (2008). Rebamipide affects the efficiency of colchicine for the herpes simplex virus-induced inflammation in a Behçet's disease mouse model. *Eur J Pharmacol, 598*(1-3), 112-117. doi:10.1016/j.ejphar.2008.09.005

Bang, D., Lee, J. H., Lee, E. S., Lee, S., Choi, J. S., Kim, Y. K., . . . Kwon, K. S. (2001). Epidemiologic and clinical survey of Behçet's disease in Korea: the first multicenter study. *J Korean Med Sci, 16*(5), 615-618.

Barra, C., Belfort Junior, R., Abreu, M. T., Kim, M. K., Martins, M. C., & Petrilli, A. M. (1991). Behçet's disease in Brazil--a review of 49 cases with emphasis on ophthalmic manifestations. *Jpn J Ophthalmol, 35*(3), 339-346.

Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J. H., Lanier, L. L., & Spies, T. (1999). Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science, 285*(5428), 727-729.

Behçet, H. (1937). Ueber Rezidivierende, aphthoese durch ein Virus verursachte Geschwuere am Mund, am Auge und an den Genitalen. *Dermatol Wochenschr, 105*, 1152-1157.

Ben Ahmed, M., Houman, H., Abdelhak, S., Ben Ghorbel, I., Miled, M., Dellagi, K., & Louzir, H. (2003). MICA transmembrane region polymorphism and HLA B51 in Tunisian Behçet's disease patients. *Adv Exp Med Biol, 528*, 225-228. doi:10.1007/0-306-48382-3_44

Benezra, D., & Cohen, E. (1986). Treatment and visual prognosis in Behçet's disease. *Br J Ophthalmol, 70*(8), 589-592.

BenEzra, D., & Nussenblatt, R. (1978). Ocular manifestations of Behçet's disease. *J Oral Pathol, 7*(6), 431-435.

Besson, C., Roetynck, S., Williams, F., Orsi, L., Amiel, C., Lependeven, C., . . . Abel, L. (2007). Association of killer cell immunoglobulin-like receptor genes with

Hodgkin's lymphoma in a familial study. *PLoS One, 2*(5), e406. doi:10.1371/journal.pone.0000406

Bjorkstrom, N. K., Beziat, V., Cichocki, F., Liu, L. L., Levine, J., Larsson, S., . . . Malmberg, K. J. (2012). CD8 T cells express randomly selected KIRs with distinct specificities compared with NK cells. *Blood, 120*(17), 3455-3465. doi:10.1182/blood-2012-03-416867

Bodmer, J. (1996). World distribution of HLA alleles and implications for disease. *Ciba Found Symp, 197*, 233-253; discussion 253-238.

Boudreau, J. E., Mulrooney, T. J., Le Luduec, J. B., Barker, E., & Hsu, K. C. (2016). KIR3DL1 and HLA-B Density and Binding Calibrate NK Education and Response to HIV. *J Immunol, 196*(8), 3398-3410. doi:10.4049/jimmunol.1502469

Boulet, S., Kleyman, M., Kim, J. Y., Kamya, P., Sharafi, S., Simic, N., . . . Bernard, N. F. (2008). A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection. *AIDS, 22*(12), 1487-1491. doi:10.1097/QAD.0b013e3282ffde7e

Bunce, M., O'Neill, C. M., Barnardo, M. C., Krausa, P., Browning, M. J., Morris, P. J., & Welsh, K. I. (1995). Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens, 46*(5), 355-367.

Burian, A., Wang, K. L., Finton, K. A., Lee, N., Ishitani, A., Strong, R. K., & Geraghty, D. E. (2016). HLA-F and MHC-I Open Conformers Bind Natural Killer Cell Ig-Like Receptor KIR3DS1. *PLoS One, 11*(9), e0163297. doi:10.1371/journal.pone.0163297

Byrne, P., McGuirk, P., Todryk, S., & Mills, K. H. (2004). Depletion of NK cells results in disseminating lethal infection with Bordetella pertussis associated with a

reduction of antigen-specific Th1 and enhancement of Th2, but not Tr1 cells. *Eur J Immunol, 34*(9), 2579-2588. doi:10.1002/eji.200425092

Cakir, N., Dervis, E., Benian, O., Pamuk, O. N., Sonmezates, N., Rahimoglu, R., . . . Sarikaya, Y. (2004). Prevalence of Behçet's disease in rural western Turkey: a preliminary report. *Clin Exp Rheumatol, 22*(4 Suppl 34), S53-55.

Calamia, K. T., Wilson, F. C., Icen, M., Crowson, C. S., Gabriel, S. E., & Kremers, H. M. (2009). Epidemiology and clinical characteristics of Behçet's disease in the US: a population-based study. *Arthritis Rheum, 61*(5), 600-604. doi:10.1002/art.24423

Caligiuri, M. A. (2008). Human natural killer cells. *Blood, 112*(3), 461-469. doi:10.1182/blood-2007-09-077438

Cambra, A., Munoz-Saa, I., Crespi, C., Serra, A., Etxagibel, A., Matamoros, N., . . . Julia, M. R. (2009). MICA-HLA-B haplotype diversity and linkage disequilibrium in a population of Jewish descent from Majorca (the Balearic Islands). *Hum Immunol, 70*(7), 513-517. doi:10.1016/j.humimm.2009.04.005

Campbell, H., & Rudan, I. (2002). Interpretation of genetic association studies in complex disease. *Pharmacogenomics J, 2*(6), 349-360. doi:10.1038/sj.tpj.6500132

Capittini, C., Pasi, A., Bergamaschi, P., Tinelli, C., De Silvestri, A., Mercati, M. P., . . . Cuccia, M. (2009). HLA haplotypes and birth weight variation: is your future going to be light or heavy? *Tissue Antigens, 74*(2), 156-163. doi:10.1111/j.1399-0039.2009.01282.x

Caporn, N., Higgs, E. R., Dieppe, P. A., & Watt, I. (1983). Arthritis in Behçet's syndrome. *Br J Radiol, 56*(662), 87-91. doi:10.1259/0007-1285-56-662-87

Carapito, R., Shahram, F., Michel, S., Le Gentil, M., Radosavljevic, M., Meguro, A., . . . Bahram, S. (2015). On the genetics of the Silk Route: association analysis of

HLA, IL10, and IL23R-IL12RB2 regions with Behçet's disease in an Iranian population. *Immunogenetics, 67*(5-6), 289-293. doi:10.1007/s00251-015-0841-6

Carlomagno, S., Falco, M., Bono, M., Alicata, C., Garbarino, L., Mazzocco, M., . . . Sivori, S. (2017). KIR3DS1-Mediated Recognition of HLA-*B51: Modulation of KIR3DS1 Responsiveness by Self HLA-B Allotypes and Effect on NK Cell Licensing. *Front Immunol, 8*, 581. doi:10.3389/fimmu.2017.00581

Cerboni, C., Neri, F., Casartelli, N., Zingoni, A., Cosman, D., Rossi, P., . . . Doria, M. (2007). Human immunodeficiency virus 1 Nef protein downmodulates the ligands of the activating receptor NKG2D and inhibits natural killer cell-mediated cytotoxicity. *J Gen Virol, 88*(Pt 1), 242-250. doi:10.1099/vir.0.82125-0

Chamberlain, M. A. (1977). Behçet's syndrome in 32 patients in Yorkshire. *Ann Rheum Dis, 36*(6), 491-499.

Cho, S. B., Sohn, S., Zheng, Z., Cho, A., Kim, H., Kang, W. J., . . . Lee, J. H. (2013). Detection of the inflammatory process in a Behçet's disease-like mouse model using 18F-fluorodeoxyglucose positron emission tomography. *Clin Exp Rheumatol, 31*(3 Suppl 77), 47-53.

Choi, B., Kim, J., Lee, E. S., Bang, D., & Sohn, S. (2011). Synthesized pyridine compound derivatives decreased TNF alpha and adhesion molecules and ameliorated HSV-induced inflammation in a mouse model. *Eur J Pharmacol, 657*(1-3), 167-172. doi:10.1016/j.ejphar.2011.01.062

Choi, B., Lee, E. S., & Sohn, S. (2011). Vitamin D3 ameliorates herpes simplex virus-induced Behçet's disease-like inflammation in a mouse model through down-regulation of Toll-like receptors. *Clin Exp Rheumatol, 29*(4 Suppl 67), S13-19.

Choukri, F., Chakib, A., Himmich, H., Hue, S., & Caillat-Zucman, S. (2001). HLA-B*51 and B*15 alleles confer predisposition to Behçet's disease in Moroccan patients. *Hum Immunol, 62*(2), 180-185.

Chung, Y. L., Bang, D. S., Lee, E. S., Lee, S. N., Mok, J. W., & Park, K. S. (2003). Behçet's disease: the first Mongolian case in literature showing HLA B51, MICA gene type *5/*6. *Yonsei Med J, 44*(5), 935-938. doi:10.3349/ymj.2003.44.5.935

Chung, Y. M., Yeh, T. S., Sheu, M. M., Chen, M. S., Wen, M. S., Tsai, H. Y., & Liao, H. R. (1990). [Behçet's disease with ocular involvement in Taiwan: a joint survey of six major ophthalmological departments]. *J Formos Med Assoc, 89*(5), 413-417.

Cohen, R., Metzger, S., Nahir, M., & Chajek-Shaul, T. (2002). Association of the MIC-A gene and HLA-B51 with Behçet's disease in Arabs and non-Ashkenazi Jews in Israel. *Ann Rheum Dis, 61*(2), 157-160.

Cooper, M. A., Fehniger, T. A., Turner, S. C., Chen, K. S., Ghaheri, B. A., Ghayur, T., . . . Caligiuri, M. A. (2001). Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood, 97*(10), 3146-3151.

Cseuz, R., & Panayi, G. S. (1990). The inhibition of NK cell function by azathioprine during the treatment of patients with rheumatoid arthritis. *Br J Rheumatol, 29*(5), 358-362.

Czeuz, R., Barnes, P., & Panayi, G. S. (1990). Natural killer cells in the blood of patients with rheumatoid arthritis treated with azathioprine. *Br J Rheumatol, 29*(4), 284-287.

Dalbeth, N., & Callan, M. F. (2002). A subset of natural killer cells is greatly expanded within inflamed joints. *Arthritis Rheum, 46*(7), 1763-1772. doi:10.1002/art.10410

Dayanc, B. E., Bansal, S., Gure, A. O., Gollnick, S. O., & Repasky, E. A. (2013). Enhanced sensitivity of colon tumour cells to natural killer cell cytotoxicity after

mild thermal stress is regulated through HSF1-mediated expression of MICA. *Int J Hyperthermia, 29*(5), 480-490. doi:10.3109/02656736.2013.821526

De Maria, A., Bozzano, F., Cantoni, C., & Moretta, L. (2011). Revisiting human natural killer cell subset function revealed cytolytic CD56(dim)CD16+ NK cells as rapid producers of abundant IFN-gamma on activation. *Proc Natl Acad Sci U S A, 108*(2), 728-732. doi:10.1073/pnas.1012356108

de Menthon, M., Lavalley, M. P., Maldini, C., Guillevin, L., & Mahr, A. (2009). HLA-B51/B5 and the risk of Behçet's disease: a systematic review and meta-analysis of case-control genetic association studies. *Arthritis Rheum, 61*(10), 1287-1296. doi:10.1002/art.24642

Diaz-Pena, R., Vidal-Castineira, J. R., Alonso-Arias, R., Suarez-Alvarez, B., Vicario, J. L., Solana, R., . . . Lopez-Larrea, C. (2010). Association of the KIR3DS1*013 and KIR3DL1*004 alleles with susceptibility to ankylosing spondylitis. *Arthritis Rheum, 62*(4), 1000-1006. doi:10.1002/art.27332

Dilsen, N., Konice, M., Aral, O., Ocal, L., Inanc, M., & Gul, A. (1993). Comparative study of the skin pathergy test with blunt and sharp needles in Behçet's disease: confirmed specificity but decreased sensitivity with sharp needles. *Ann Rheum Dis, 52*(11), 823-825.

Direskeneli, H., Eksioglu-Demiralp, E., Yavuz, S., Ergun, T., Shinnick, T., Lehner, T., & Akoglu, T. (2000). T cell responses to 60/65 kDa heat shock protein derived peptides in Turkish patients with Behçet's disease. *J Rheumatol, 27*(3), 708-713.

Disease, I. S. G. f. t. d. o. B. s. (1990). Criteria for diagnosis of Behçet's disease. International Study Group for Behçet's Disease. *Lancet, 335*(8697), 1078-1080.

Dorner, B. G., Scheffold, A., Rolph, M. S., Huser, M. B., Kaufmann, S. H., Radbruch, A., . . . Kroczek, R. A. (2002). MIP-1alpha, MIP-1beta, RANTES, and

ATAC/lymphotactin function together with IFN-gamma as type 1 cytokines. *Proc Natl Acad Sci U S A, 99*(9), 6181-6186. doi:10.1073/pnas.092141999

Duzgun, N., Ates, A., Aydintug, O. T., Demir, O., & Olmez, U. (2006). Characteristics of vascular involvement in Behçet's disease. *Scand J Rheumatol, 35*(1), 65-68. doi:10.1080/03009740500255761

Eglin, R. P., Lehner, T., & Subak-Sharpe, J. H. (1982). Detection of RNA complementary to herpes-simplex virus in mononuclear cells from patients with Behçet's syndrome and recurrent oral ulcers. *Lancet, 2*(8312), 1356-1361.

Eksioglu-Demiralp, E., Direskeneli, H., Ergun, T., Fresko, I., & Akoglu, T. (1999). Increased CD4+CD16+ and CD4+CD56+ T cell subsets in Behçet's disease. *Rheumatol Int, 19*(1-2), 23-26.

El Menyawi, M. M., Raslan, H. M., & Edrees, A. (2009). Clinical features of Behçet's disease in Egypt. *Rheumatol Int, 29*(6), 641-646. doi:10.1007/s00296-008-0741-2

Erer, B., Takeuchi, M., Ustek, D., Tugal-Tutkun, I., Seyahi, E., Ozyazgan, Y., . . . Ombrello, M. J. (2016). Evaluation of KIR3DL1/KIR3DS1 polymorphism in Behçet's disease. *Genes Immun, 17*(7), 396-399. doi:10.1038/gene.2016.36

Ergun, T., Ince, U., Eksioglu-Demiralp, E., Direskeneli, H., Gurbuz, O., Gurses, L., . . . Akoglu, T. (2001). HSP 60 expression in mucocutaneous lesions of Behçet's disease. *J Am Acad Dermatol, 45*(6), 904-909. doi:10.1067/mjd.2001.117728

Eyerci, N., Akdeniz, N., & Keles, S. (2018). Association of MICA Alleles and Human Leukocyte Antigen B in Turkish Patients Diagnosed With Behçet's Disease. *Archives of rheumatology, **33***(3), 352-357.

Fatemi, A., Shahram, F., Akhlaghi, M., Smiley, A., Nadji, A., & Davatchi, F. (2015). Prospective study of articular manifestations in Behçet's disease: five-year report. *Int J Rheum Dis*. doi:10.1111/1756-185X.12633

Faure, M., & Long, E. O. (2002). KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential. *J Immunol, 168*(12), 6208-6214.

Fei, Y., Li, X., Lin, S., Song, X., Wu, Q., Zhu, Y., . . . Zhang, F. (2013). Major vascular involvement in Behçet's disease: a retrospective study of 796 patients. *Clin Rheumatol, 32*(6), 845-852. doi:10.1007/s10067-013-2205-7

Fei, Y., Webb, R., Cobb, B. L., Direskeneli, H., Saruhan-Direskeneli, G., & Sawalha, A. H. (2009). Identification of novel genetic susceptibility loci for Behçet's disease using a genome-wide association study. *Arthritis Res Ther, 11*(3), R66. doi:10.1186/ar2695

Fietta, P. (2005). Behçet's disease: familial clustering and immunogenetics. *Clin Exp Rheumatol, 23*(4 Suppl 38), S96-105.

Fodil, N., Pellet, P., Laloux, L., Hauptmann, G., Theodorou, I., & Bahram, S. (1999). MICA haplotypic diversity. *Immunogenetics, 49*(6), 557-560.

French, A. R., & Yokoyama, W. M. (2004). Natural killer cells and autoimmunity. *Arthritis Res Ther, 6*(1), 8-14. doi:10.1186/ar1034

Frigoul, A., & Lefranc, M. (2005). MICA: standardized IMGT allele nomenclature, polymorphisms and diseases. In S. Pandalai (Ed.), *Recent Research Developments in Human Genetics* (Vol. 3, pp. 95–145). Trivandrum, Kerala, India: Research Signpost.

Furukawa, H., Yabe, T., Watanabe, K., Miyamoto, R., Miki, A., Akaza, T., . . . Juji, T. (1999). Tolerance of NK and LAK activity for HLA class I-deficient targets in a TAP1-deficient patient (bare lymphocyte syndrome type I). *Hum Immunol, 60*(1), 32-40.

Gao, X., Single, R. M., Karacki, P., Marti, D., O'Brien, S. J., & Carrington, M. (2006). Diversity of MICA and linkage disequilibrium with HLA-B in two North

American populations. *Hum Immunol, 67*(3), 152-158. doi:10.1016/j.humimm.2006.02.009

Garcia-Beltran, W. F., Holzemer, A., Martrus, G., Chung, A. W., Pacheco, Y., Simoneau, C. R., . . . Altfeld, M. (2016). Open conformers of HLA-F are high-affinity ligands of the activating NK-cell receptor KIR3DS1. *Nat Immunol, 17*(9), 1067-1074. doi:10.1038/ni.3513

Gardiner, C. M., Guethlein, L. A., Shilling, H. G., Pando, M., Carr, W. H., Rajalingam, R., . . . Parham, P. (2001). Different NK cell surface phenotypes defined by the DX9 antibody are due to KIR3DL1 gene polymorphism. *J Immunol, 166*(5), 2992-3001.

Gebreselassie, D., Spiegel, H., & Vukmanovic, S. (2006). Sampling of major histocompatibility complex class I-associated peptidome suggests relatively looser global association of HLA-B*5101 with peptides. *Hum Immunol, 67*(11), 894-906. doi:10.1016/j.humimm.2006.08.294

Ghadially, H., Brown, L., Lloyd, C., Lewis, L., Lewis, A., Dillon, J., . . . Wilkinson, R. W. (2017). MHC class I chain-related protein A and B (MICA and MICB) are predominantly expressed intracellularly in tumour and normal tissue. *Br J Cancer, 116*(9), 1208-1217. doi:10.1038/bjc.2017.79

Giza, M., Koftori, D., Chen, L., & Bowness, P. (2018). Is Behçet's disease a 'class 1-opathy'? The role of HLA-B*51 in the pathogenesis of Behçet's disease. *Clin Exp Immunol, 191*(1), 11-18. doi:10.1111/cei.13049

Gonzalez, S., Brautbar, C., Martinez-Borra, J., Lopez-Vazquez, A., Segal, R., Blanco-Gelaz, M. A., . . . Lopez-Larrea, C. (2001). Polymorphism in MICA rather than HLA-B/C genes is associated with psoriatic arthritis in the Jewish population. *Hum Immunol, 62*(6), 632-638.

Gonzalez-Escribano, M. F., Rodriguez, M. R., Aguilar, F., Alvarez, A., Sanchez-Roman, J., & Nunez-Roldan, A. (1999). Lack of association of MICA transmembrane region polymorphism and Behçet's disease in Spain. *Tissue Antigens, 54*(3), 278-281.

González-Gay, M. A., García-Porrúa, C., Brañas, F., López-Lázaro, L., & Olivieri, I. (2000). Epidemiologic and clinical aspects of Behçet's disease in a defined area of Northwestern Spain, 1988-1997. *The Journal of rheumatology, 27*(3), 703-707.

Goodridge, J. P., Burian, A., Lee, N., & Geraghty, D. E. (2010). HLA-F complex without peptide binds to MHC class I protein in the open conformer form. *J Immunol, 184*(11), 6199-6208. doi:10.4049/jimmunol.1000078

Goto, K., & Kato, N. (2015). MICA SNPs and the NKG2D system in virus-induced HCC. *J Gastroenterol, 50*(3), 261-272. doi:10.1007/s00535-014-1000-9

Goto, K., Ota, M., Ohno, S., Mizuki, N., Ando, H., Katsuyama, Y., . . . Inoko, H. (1997). MICA gene and ankylosing spondylitis: linkage analysis via a transmembrane-encoded triplet repeat polymorphism. *Tissue Antigens, 49*(5), 503-507.

Groh, V., Wu, J., Yee, C., & Spies, T. (2002). Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature, 419*(6908), 734-738. doi:10.1038/nature01112

Guasp, P., Barnea, E., Gonzalez-Escribano, M. F., Jimenez-Reinoso, A., Regueiro, J. R., Admon, A., & Lopez de Castro, J. A. (2017). The Behçet's disease-associated variant of the aminopeptidase ERAP1 shapes a low affinity HLA-B*51 peptidome by differential subpeptidome processing. *J Biol Chem*. doi:10.1074/jbc.M117.789180

Guethlein, L. A., Norman, P. J., Hilton, H. H., & Parham, P. (2015). Co-evolution of MHC class I and variable NK cell receptors in placental mammals. *Immunol Rev, 267*(1), 259-282. doi:10.1111/imr.12326

Gul, A. (2011). HLA-B51 and Behçet's Disease. *Ocular Immunology and Inflammation*.

Gul, A., Inanc, M., Ocal, L., Aral, O., & Konice, M. (2000). Familial aggregation of Behçet's disease in Turkey. *Ann Rheum Dis, 59*(8), 622-625.

Gul, A., Tugal-Tutkun, I., Dinarello, C. A., Reznikov, L., Esen, B. A., Mirza, A., . . . Solinger, A. (2012). Interleukin-1beta-regulating antibody XOMA 052 (gevokizumab) in the treatment of acute exacerbations of resistant uveitis of Behçet's disease: an open-label pilot study. *Ann Rheum Dis, 71*(4), 563-566. doi:10.1136/annrheumdis-2011-155143

Gul, A., Uyar, F. A., Inanc, M., Ocal, L., Barrett, J. H., Aral, O., . . . Saruhan-Direskeneli, G. (2002). A weak association of HLA-B*2702 with Behçet's disease. *Genes Immun, 3*(6), 368-372. doi:10.1038/sj.gene.6363863

Gul, A., Uyar, F. A., Inanc, M., Ocal, L., Tugal-Tutkun, I., Aral, O., . . . Saruhan-Direskeneli, G. (2001). Lack of association of HLA-B*51 with a severe disease course in Behçet's disease. *Rheumatology (Oxford), 40*(6), 668-672.

Gumperz, J. E., Barber, L. D., Valiante, N. M., Percival, L., Phillips, J. H., Lanier, L. L., & Parham, P. (1997). Conserved and variable residues within the Bw4 motif of HLA-B make separable contributions to recognition by the NKB1 killer cell-inhibitory receptor. *J Immunol, 158*(11), 5237-5241.

Gumperz, J. E., Litwin, V., Phillips, J. H., Lanier, L. L., & Parham, P. (1995). The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med, 181*(3), 1133-1144.

Gumperz, J. E., Valiante, N. M., Parham, P., Lanier, L. L., & Tyan, D. (1996). Heterogeneous phenotypes of expression of the NKB1 natural killer cell class I receptor among individuals of different human histocompatibility leukocyte antigens types appear genetically regulated, but not linked to major histocompatibililty complex haplotype. *J Exp Med, 183*(4), 1817-1827.

Gur, C., Porgador, A., Elboim, M., Gazit, R., Mizrahi, S., Stern-Ginossar, N., . . . Mandelboim, O. (2010). The activating receptor NKp46 is essential for the development of type 1 diabetes. *Nat Immunol, 11*(2), 121-128. doi:10.1038/ni.1834

Haldane, J. B. (1945). The use of chi 2 as a test of homogeniety in a (mu chi 2)-fold table when expectations are small. *Biometrika, 33*, 234-238.

Hamza, M., Elleuch, M., Slim, A., Hamzaoui, K., & Ayed, K. (1990). Antibodies to herpes simplex virus in patients with Behçet's disease. *Clin Rheumatol, 9*(4), 498-500.

Hamza, M., & Slim, A. (1991). Herpes simplex virus antigens in sera of patients with Behçet's disease. *Clin Rheumatol, 10*(4), 456.

Hamzaoui, K., Ayed, K., Hamza, M., & Touraine, J. L. (1988). Natural killer cells in Behçet's disease. *Clin Exp Immunol, 71*(1), 126-131.

Hamzaoui, K., Ayed, K., Slim, A., Hamza, M., & Touraine, J. (1990). Natural killer cell activity, interferon-gamma and antibodies to herpes viruses in patients with Behçet's disease. *Clin Exp Immunol, 79*(1), 28-34.

Hamzaoui, K., & Ben Ammar, A. G. (1992). [Herpes simplex virus in Behçet's disease and in systemic diseases]. *Tunis Med, 70*(3), 127-129.

Hamzaoui, K., Berraies, A., Kaabachi, W., Ammar, J., & Hamzaoui, A. (2013). Pulmonary manifestations in Behçet disease: impaired natural killer cells activity. *Multidiscip Respir Med, 8*(1), 29. doi:10.1186/2049-6958-8-29

Hasan, M. S., Ryan, P. L., Bergmeier, L. A., & Fortune, F. (2017). Circulating NK cells and their subsets in Behçet's Disease. *Clin Exp Immunol*. doi:10.1111/cei.12939

Hatemi, G., Silman, A., Bang, D., Bodaghi, B., Chamberlain, A. M., Gul, A., . . . Committee, E. E. (2008). EULAR recommendations for the management of Behçet disease. *Ann Rheum Dis, 67*(12), 1656-1662. doi:10.1136/ard.2007.080432

Hirohata, S., & Hashimoto, T. (1998). Abnormal T cell responses to bacterial superantigens in Behçet's disease (BD). *Clin Exp Immunol, 112*(2), 317-324.

Hirohata, S., Oka, H., & Mizushima, Y. (1992). Streptococcal-related antigens stimulate production of IL6 and interferon-gamma by T cells from patients with Behçet's disease. *Cell Immunol, 140*(2), 410-419.

Hirschberg, J. (1899). Geschichte der Augenheilkunde *Graefe-Saemisch Handbuch der gesamten Augenheilkunde* (2 ed., pp. 70). Leipzig: Engelmann.

Hollenbach, J. A., Pando, M. J., Caillier, S. J., Gourraud, P. A., & Oksenberg, J. R. (2016). The killer immunoglobulin-like receptor KIR3DL1 in combination with HLA-Bw4 is protective against multiple sclerosis in African Americans. *Genes Immun, 17*(3), 199-202. doi:10.1038/gene.2016.5

Hou, S., Yang, Z., Du, L., Jiang, Z., Shu, Q., Chen, Y., . . . Yang, P. (2012). Identification of a susceptibility locus in STAT4 for Behçet's disease in Han Chinese in a genome-wide association study. *Arthritis Rheum, 64*(12), 4104-4113. doi:10.1002/art.37708

Hughes, E. H., Collins, R. W., Kondeatis, E., Wallace, G. R., Graham, E. M., Vaughan, R. W., & Stanford, M. R. (2005). Associations of major histocompatibility complex class I chain-related molecule polymorphisms with Behçet's disease in Caucasian patients. *Tissue Antigens, 66*(3), 195-199. doi:10.1111/j.1399-0039.2005.00465.x

Hughes, T., Coit, P., Adler, A., Yilmaz, V., Aksu, K., Duzgun, N., . . . Sawalha, A. H. (2013). Identification of multiple independent susceptibility loci in the HLA region in Behçet's disease. *Nat Genet, 45*(3), 319-324. doi:10.1038/ng.2551

Ikeda, N., Kojima, H., Nishikawa, M., Hayashi, K., Futagami, T., Tsujino, T., . . . Saji, H. (2015). Determination of HLA-A, -C, -B, -DRB1 allele and haplotype frequency in Japanese population based on family study. *Tissue Antigens, 85*(4), 252-259. doi:10.1111/tan.12536

Ilter, N., Senol, E., Gurer, M. A., & Oztas, M. O. (2000). Behçet's disease and HCV infection. *Int J Dermatol, 39*(5), 396-397.

Inngjerdingen, M., Kveberg, L., Naper, C., & Vaage, J. T. (2011). Natural killer cell subsets in man and rodents. *Tissue Antigens, 78*(2), 81-88. doi:10.1111/j.1399-0039.2011.01714.x

International Team for the Revision of the International Criteria for Behçet's, D. (2014). The International Criteria for Behçet's Disease (ICBD): a collaborative study of 27 countries on the sensitivity and specificity of the new criteria. *J Eur Acad Dermatol Venereol, 28*(3), 338-347. doi:10.1111/jdv.12107

ISG. (1990). Criteria for diagnosis of Behçet's disease. International Study Group for Behçet's Disease. *Lancet, 335*(8697), 1078-1080.

Jankowski, J., Crombie, I., & Jankowski, R. (1992). Behçet's syndrome in Scotland. *Postgrad Med J, 68*(801), 566-570.

Japan, T. B. s. D. R. C. o. (1989). Skin hypersensitivity to streptococcal antigens and the induction of systemic symptoms by the antigens in Behçet's disease--a multicenter study. The Behçet's Disease Research Committee of Japan. *Journal of Rheumatology, 16*(4), 506-511.

Kaburaki, T., Takamoto, M., Numaga, J., Kawashima, H., Araie, M., Ohnogi, Y., . . . Takeuchi, F. (2010). Genetic association of HLA-A*2601 with ocular Behçet's disease in Japanese patients. *Clin Exp Rheumatol, 28*(4 Suppl 60), S39-44.

Kaneko, F., Takahashi, Y., Muramatsu, R., Adachi, K., Miura, Y., Nakane, A., & Minagawa, T. (1985). Natural killer cell numbers and function in peripheral lymphoid cells in Behçet's disease. *Br J Dermatol, 113*(3), 313-318.

Kaneko, S., Suzuki, N., Yamashita, N., Nagafuchi, H., Nakajima, T., Wakisaka, S., . . . Sakane, T. (1997). Characterization of T cells specific for an epitope of human 60-kD heat shock protein (hsp) in patients with Behçet's disease (BD) in Japan. *Clin Exp Immunol, 108*(2), 204-212.

Kappen, J. H., Medina-Gomez, C., van Hagen, P. M., Stolk, L., Estrada, K., Rivadeneira, F., . . . van Laar, J. A. (2015). Genome-wide association study in an admixed case series reveals IL12A as a new candidate in Behçet disease. *PLoS One, 10*(3), e0119085. doi:10.1371/journal.pone.0119085

Karre, K., Ljunggren, H. G., Piontek, G., & Kiessling, R. (1986). Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature, 319*(6055), 675-678. doi:10.1038/319675a0

Kato, K., Hasegawa, R., Katsuta, T., Taniguchi, H., Kondo, Y., Kimura, T., . . . Yokoyama, T. (2006). [Case of acute respiratory distress syndrome in which high-frequency oscillatory ventilation (HFOV) was effective for the management of severe hypoxemia]. *Nihon Kokyuki Gakkai Zasshi, 44*(10), 732-737.

Kato, N., Tanaka, J., Sugita, J., Toubai, T., Miura, Y., Ibata, M., . . . Imamura, M. (2007). Regulation of the expression of MHC class I-related chain A, B (MICA, MICB) via chromatin remodeling and its impact on the susceptibility of

leukemic cells to the cytotoxicity of NKG2D-expressing cells. *Leukemia, 21*(10), 2103-2108. doi:10.1038/sj.leu.2404862

Katz, G., Markel, G., Mizrahi, S., Arnon, T. I., & Mandelboim, O. (2001). Recognition of HLA-Cw4 but not HLA-Cw6 by the NK cell receptor killer cell Ig-like receptor two-domain short tail number 4. *J Immunol, 166*(12), 7260-7267.

Katzenellenbogen, I., & Feuerman, E. J. (1965). [Data on Behçet's Disease. (Significance of the Specific Skin Hyperreactivity and of the Behçetin Reaction)]. *Hautarzt, 16*, 13-18.

Kaufmann, S. H., Schoel, B., van Embden, J. D., Koga, T., Wand-Wurttenberger, A., Munk, M. E., & Steinhoff, U. (1991). Heat-shock protein 60: implications for pathogenesis of and protection against bacterial infections. *Immunol Rev, 121*, 67-90.

Keogan, M. T. (2009). Clinical Immunology Review Series: an approach to the patient with recurrent orogenital ulceration, including Behçet's syndrome. *Clin Exp Immunol, 156*(1), 1-11. doi:10.1111/j.1365-2249.2008.03857.x

Kera, J., Mizuki, N., Ota, M., Katsuyama, Y., Pivetti-Pezzi, P., Ohno, S., & Inoko, H. (1999). Significant associations of HLA-B*5101 and B*5108, and lack of association of class II alleles with Behçet's disease in Italian patients. *Tissue Antigens, 54*(6), 565-571.

Kiani, Z., Dupuy, F. P., Bruneau, J., Lebouche, B., Zhang, C. X., Jackson, E., . . . Bernard, N. F. (2018). HLA-F on HLA-Null 721.221 Cells Activates Primary NK Cells Expressing the Activating Killer Ig-like Receptor KIR3DS1. *J Immunol, 201*(1), 113-123. doi:10.4049/jimmunol.1701370

Kim do, Y., Cho, S., Choi, M. J., Sohn, S., Lee, E. S., & Bang, D. (2013). Immunopathogenic Role of Herpes Simplex Virus in Behçet's Disease. *Genet Res Int, 2013*, 638273. doi:10.1155/2013/638273

Kimura, M. Y., & Nakayama, T. (2005). Differentiation of NK1 and NK2 cells. *Crit Rev Immunol, 25*(5), 361-374.

Kiraz, S., Ozturk, M. A., Ertenli, I., & Calguneri, M. (2001). Parvovirus B19 infection in Behçet's disease. *Ann Rheum Dis, 60*(8), 814-815.

Kirino, Y., Bertsias, G., Ishigatsubo, Y., Mizuki, N., Tugal-Tutkun, I., Seyahi, E., . . . Kastner, D. L. (2013). Genome-wide association analysis identifies new susceptibility loci for Behçet's disease and epistasis between HLA-B*51 and ERAP1. *Nat Genet, 45*(2), 202-207. doi:10.1038/ng.2520

Kone-Paut, I., Geisler, I., Wechsler, B., Ozen, S., Ozdogan, H., Rozenbaum, M., & Touitou, I. (1999). Familial aggregation in Behçet's disease: high frequency in siblings and parents of pediatric probands. *J Pediatr, 135*(1), 89-93.

Kotter, I., Gunaydin, I., Stubiger, N., Yazici, H., Fresko, I., Zouboulis, C. C., . . . Muller, C. A. (2001). Comparative analysis of the association of HLA-B*51 suballeles with Behçet's disease in patients of German and Turkish origin. *Tissue Antigens, 58*(3), 166-170.

Krause, I., Yankevich, A., Fraser, A., Rosner, I., Mader, R., Zisman, D., . . . Weinberger, A. (2007). Prevalence and clinical aspects of Behçet's disease in the north of Israel. *Clin Rheumatol, 26*(4), 555-560. doi:10.1007/s10067-006-0349-4

Krause, L., Kohler, A. K., Altenburg, A., Papoutsis, N., Zouboulis, C. C., Pleyer, U., . . . Foerster, M. H. (2009). Ocular involvement is associated with HLA-B51 in Adamantiades-Behçet's disease. *Eye (Lond), 23*(5), 1182-1186. doi:10.1038/eye.2008.177

Kuzu, M. A., Ozaslan, C., Koksoy, C., Gurler, A., & Tuzuner, A. (1994). Vascular involvement in Behçet's disease: 8-year audit. *World J Surg, 18*(6), 948-953; discussion 953-944.

Lanier, L. L. (1998). NK cell receptors. *Annu Rev Immunol, 16*, 359-393. doi:10.1146/annurev.immunol.16.1.359

Lee, N., Ishitani, A., & Geraghty, D. E. (2010). HLA-F is a surface marker on activated lymphocytes. *Eur J Immunol, 40*(8), 2308-2318.

Lee, S. (2001). *Behçet's disease : a guide to its clinical understanding : textbook and atlas*. Berlin ; New York: Springer.

Lee, S., Bang, D., Cho, Y. H., Lee, E. S., & Sohn, S. (1996). Polymerase chain reaction reveals herpes simplex virus DNA in saliva of patients with Behçet's disease. *Arch Dermatol Res, 288*(4), 179-183.

Lee, Y. B., Lee, S. Y., Choi, J. Y., Lee, J. H., Chae, H. S., Kim, J. W., . . . Yu, D. S. (2017). Incidence, prevalence, and mortality of Adamantiades-Behçet's disease in Korea: a nationwide, population-based study (2006-2015). *J Eur Acad Dermatol Venereol*. doi:10.1111/jdv.14601

Lee, Y. H., & Song, G. G. (2015). Associations between major histocompatibility complex class I chain-related gene A polymorphisms and susceptibility to Behçet's disease : A meta-analysis. *Z Rheumatol, 74*(8), 714-721. doi:10.1007/s00393-014-1536-3

Lee, Y. J., Horie, Y., Wallace, G. R., Choi, Y. S., Park, J. A., Choi, J. Y., . . . Lee, E. B. (2013). Genome-wide association study identifies GIMAP as a novel susceptibility locus for Behçet's disease. *Ann Rheum Dis, 72*(9), 1510-1516. doi:10.1136/annrheumdis-2011-200288

Lefranc, M. P., Clement, O., Kaas, Q., Duprat, E., Chastellan, P., Coelho, I., . . . Lefranc, G. (2005). IMGT-Choreography for immunogenetics and immunoinformatics. *In Silico Biol, 5*(1), 45-60.

Lehner, T. (1977). Oral ulceration and Behçet's syndrome. *Gut, 18*(6), 491-511.

Lehner, T. (1997). The role of heat shock protein, microbial and autoimmune agents in the aetiology of Behçet's disease. *Int Rev Immunol, 14*(1), 21-32. doi:10.3109/08830189709116842

Lehner, T., Barnes, C. G., & Royal Society of Medicine (Great Britain). (1979). *Behçet's syndrome : clinical and immunological features : proceedings of a conference sponsored by the Royal Society of Medicine, February, 1979*. London ; New York: Academic Press.

Lehner, T., Lavery, E., Smith, R., van der Zee, R., Mizushima, Y., & Shinnick, T. (1991). Association between the 65-kilodalton heat shock protein, Streptococcus sanguis, and the corresponding antibodies in Behçet's syndrome. *Infect Immun, 59*(4), 1434-1441.

Li, H., Pascal, V., Martin, M. P., Carrington, M., & Anderson, S. K. (2008). Genetic control of variegated KIR gene expression: polymorphisms of the bi-directional KIR3DL1 promoter are associated with distinct frequencies of gene expression. *PLoS Genet, 4*(11), e1000254. doi:10.1371/journal.pgen.1000254

Lopalco, G., Cantarini, L., Vitale, A., Iannone, F., Anelli, M. G., Andreozzi, L., . . . Rigante, D. (2015). Interleukin-1 as a common denominator from autoinflammatory to autoimmune disorders: premises, perils, and perspectives. *Mediators Inflamm, 2015*, 194864. doi:10.1155/2015/194864

Lucas, D., Campillo, J. A., Lopez-Hernandez, R., Martinez-Garcia, P., Lopez-Sanchez, M., Botella, C., . . . Muro, M. (2008). Allelic diversity of MICA gene and MICA/HLA-B haplotypic variation in a population of the Murcia region in southeastern Spain. *Hum Immunol, 69*(10), 655-660. doi:10.1016/j.humimm.2008.07.011

Luo, J., Tian, W., Pan, F., Liu, X., & Li, L. (2014). Allelic and haplotypic diversity of 5'promoter region of the MICA gene. *Hum Immunol, 75*(4), 383-388. doi:10.1016/j.humimm.2013.12.010

Mahr, A., Belarbi, L., Wechsler, B., Jeanneret, D., Dhote, R., Fain, O., . . . Guillevin, L. (2008). Population-based prevalence study of Behçet's disease: differences by ethnic origin and low variation by age at immigration. *Arthritis Rheum, 58*(12), 3951-3959. doi:10.1002/art.24149

Marchini, J., Cardon, L. R., Phillips, M. S., & Donnelly, P. (2004). The effects of human population structure on large genetic association studies. *Nat Genet, 36*(5), 512-517. doi:10.1038/ng1337

Marin, M. L., Savioli, C. R., Yamamoto, J. H., Kalil, J., & Goldberg, A. C. (2004). MICA polymorphism in a sample of the Sao Paulo population, Brazil. *Eur J Immunogenet, 31*(2), 63-71. doi:10.1111/j.1365-2370.2004.00446.x

Martin, M. P., Qi, Y., Gao, X., Yamada, E., Martin, J. N., Pereyra, F., . . . Carrington, M. (2007). Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet, 39*(6), 733-740. doi:10.1038/ng2035

McCarthy, N. E., Hedin, C. R., Sanders, T. J., Amon, P., Hoti, I., Ayada, I., . . . Stagg, A. J. (2015). Azathioprine therapy selectively ablates human Vdelta2(+) T cells in Crohn's disease. *J Clin Invest, 125*(8), 3215-3225. doi:10.1172/JCI80840

Meguro, A., Inoko, H., Ota, M., Katsuyama, Y., Oka, A., Okada, E., . . . Mizuki, N. (2010). Genetics of Behçet disease inside and outside the MHC. *Ann Rheum Dis, 69*(4), 747-754. doi:10.1136/ard.2009.108571

Mellado, F., Talesnik, E., & Castiglione, E. (2012). Azathioprine as monotherapy for scleritis in relapsing polychondritis. *Ocul Immunol Inflamm, 20*(3), 235-236. doi:10.3109/09273948.2012.659369

Menozzi, P., Piazza, A., & Cavalli-Sforza, L. (1978). Synthetic maps of human gene frequencies in Europeans. *Science, 201*(4358), 786-792.

Middleton, D., Meenagh, A., Sleator, C., Gourraud, P. A., Ayna, T., Tozkir, H., . . . Diler, A. S. (2007). No association of KIR genes with Behçet's disease. *Tissue Antigens, 70*(5), 435-438. doi:10.1111/j.1399-0039.2007.00929.x

Mineshita, S., Tian, D., Wang, L. M., Jian, X. Y., Li, S. Y., Fang, G. Z., . . . et al. (1992). Histocompatibility antigens associated with Behçet's disease in northern Han Chinese. *Intern Med, 31*(9), 1073-1075.

Mizuki, N., Inoko, H., & Ohno, S. (1997). Molecular genetics (HLA) of Behçet's disease. *Yonsei Med J, 38*(6), 333-349.

Mizuki, N., Meguro, A., Ota, M., Ohno, S., Shiota, T., Kawagoe, T., . . . Inoko, H. (2010). Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behçet's disease susceptibility loci. *Nat Genet, 42*(8), 703-706. doi:10.1038/ng.624

Mizuki, N., Ohno, S., Ando, H., Chen, L., Palimeris, G. D., Stavropoulos-Ghiokas, E., . . . Inoko, H. (1997). A strong association between HLA-B*5101 and Behçet's disease in Greek patients. *Tissue Antigens, 50*(1), 57-60.

Mizuki, N., Ota, M., Katsuyama, Y., Yabuki, K., Ando, H., Goto, K., . . . Inoko, H. (1999). Association analysis between the MIC-A and HLA-B alleles in Japanese patients with Behçet's disease. *Arthritis Rheum, 42*(9), 1961-1966. doi:10.1002/1529-0131(199909)42:9<1961::AID-ANR23>3.0.CO;2-7

Mizuki, N., Ota, M., Kimura, M., Ohno, S., Ando, H., Katsuyama, Y., . . . Inoko, H. (1997). Triplet repeat polymorphism in the transmembrane region of the MICA gene: a strong association of six GCT repetitions with Behçet disease. *Proc Natl Acad Sci U S A, 94*(4), 1298-1303.

Mizuki, N., Ota, M., Yabuki, K., Katsuyama, Y., Ando, H., Palimeris, G. D., . . . Inoko, H. (2000). Localization of the pathogenic gene of Behçet's disease by microsatellite analysis of three different populations. *Invest Ophthalmol Vis Sci, 41*(12), 3702-3708.

Moon, S. J., Oh, E. J., Kim, Y., Kim, K. S., Kwok, S. K., Ju, J. H., . . . Park, S. H. (2013). Diversity of killer cell immunoglobulin-like receptor genes in uveitis associated with autoimmune diseases: ankylosing spondylitis and Behçet disease. *Ocul Immunol Inflamm, 21*(2), 135-143. doi:10.3109/09273948.2012.754905

Mizuki, N., Yabuki, K., Ota, M., Verity, D., Katsuyama, Y., Ando, H., . . . Inoko, H. (2001). Microsatellite mapping of a susceptible locus within the HLA region for Behçet's disease using Jordanian patients. *Hum Immunol, 62*(2), 186-190.

Mochizuki, M., Suzuki, N., Takeno, M., Nagafuchi, H., Harada, T., Kaneoka, H., . . . et al. (1994). Fine antigen specificity of human gamma delta T cell lines (V gamma 9+) established by repetitive stimulation with a serotype (KTH-1) of a gram-positive bacterium, Streptococcus sanguis. *Eur J Immunol, 24*(7), 1536-1543. doi:10.1002/eji.1830240712

Mohammad, A., Mandl, T., Sturfelt, G., & Segelmark, M. (2013). Incidence, prevalence and clinical characteristics of Behçet's disease in southern Sweden. *Rheumatology (Oxford), 52*(2), 304-310. doi:10.1093/rheumatology/kes249

Monacelli, M., & Nazzaro, P. (1966). *Behçet's disease*. Basel, New York,: S. Karger.

Montes-Cano, M. A., Conde-Jaldon, M., Garcia-Lozano, J. R., Ortiz-Fernandez, L., Ortego-Centeno, N., Castillo-Palma, M. J., . . . Gonzalez-Escribano, M. F. (2013). HLA and non-HLA genes in Behçet's disease: a multicentric study in the Spanish population. *Arthritis Res Ther, 15*(5), R145. doi:10.1186/ar4328

Morvan, M., Willem, C., Gagne, K., Kerdudou, N., David, G., Sebille, V., . . . Retiere, C. (2009). Phenotypic and functional analyses of KIR3DL1+ and KIR3DS1+ NK cell subsets demonstrate differential regulation by Bw4 molecules and induced KIR3DS1 expression on stimulated NK cells. *J Immunol, 182*(11), 6727-6735. doi:10.4049/jimmunol.0900212

Mumcu, G., Niazi, S., Stewart, J., Hagi-Pavli, E., Gokani, B., Seoudi, N., . . . Direskeneli, H. (2009). Oral health and related quality of life status in patients from UK and Turkey: a comparative study in Behçet's disease. *J Oral Pathol Med, 38*(5), 406-409. doi:10.1111/j.1600-0714.2009.00752.x

Norman, P. J., Abi-Rached, L., Gendzekhadze, K., Korbel, D., Gleimer, M., Rowley, D., . . . Parham, P. (2007a). Unusual selection on the KIR3DL1/S1 natural killer cell receptor in Africans. *Nat Genet, 39*(9), 1092-1099. doi:10.1038/ng2111

Norman, P. J., Abi-Rached, L., Gendzekhadze, K., Korbel, D., Gleimer, M., Rowley, D., . . . Parham, P. (2007b). Unusual selection on the KIR3DL1/S1 natural killer cell receptor in Africans. *Nature Genetics, 39*(9), 1092-1099. doi:Doi 10.1038/Ng2111

Norman, P. J., Hollenbach, J. A., Nemat-Gorgani, N., Marin, W. M., Norberg, S. J., Ashouri, E., . . . Parham, P. (2016). Defining KIR and HLA Class I Genotypes at Highest Resolution via High-Throughput Sequencing. *Am J Hum Genet, 99*(2), 375-391. doi:10.1016/j.ajhg.2016.06.023

Norris, S., Kondeatis, E., Collins, R., Satsangi, J., Clare, M., Chapman, R., . . . Donaldson, P. (2001). Mapping MHC-encoded susceptibility and resistance in primary sclerosing cholangitis: the role of MICA polymorphism. *Gastroenterology, 120*(6), 1475-1482.

O'Connor, G. M., Vivian, J. P., Gostick, E., Pymm, P., Lafont, B. A., Price, D. A., . . . McVicar, D. W. (2015). Peptide-Dependent Recognition of HLA-B*57:01 by KIR3DS1. *J Virol, 89*(10), 5213-5221. doi:10.1128/JVI.03586-14

O'Duffy, J. D., Taswell, H. F., & Elveback, L. R. (1976). HL-A antigens in Behçet's disease. *J Rheumatol, 3*(1), 1-3.

Ohno, S., Aoki, K., Sugiura, S., Nakayama, E., & Itakura, K. (1973). Letter: HL-A5 and Behçet's disease. *Lancet, 2*(7842), 1383-1384.

Ohno, S., Ohguchi, M., Hirose, S., Matsuda, H., Wakisaka, A., & Aizawa, M. (1982). Close association of HLA-Bw51 with Behçet's disease. *Arch Ophthalmol, 100*(9), 1455-1458.

Oliveira, L. A., Ribas, F., Bicalho, M. G., Tsuneto, L. T., & Petzl-Erler, M. L. (2008). High frequencies of alleles MICA*020 and MICA*027 in Amerindians and evidence of positive selection on exon 3. *Genes Immun, 9*(8), 697-705. doi:10.1038/gene.2008.65

Ombrello, M. J., Kirino, Y., de Bakker, P. I., Gul, A., Kastner, D. L., & Remmers, E. F. (2014). Behçet disease-associated MHC class I residues implicate antigen binding and regulation of cell-mediated cytotoxicity. *Proc Natl Acad Sci U S A, 111*(24), 8867-8872. doi:10.1073/pnas.1406575111

Ombrello, M. J., Sikora, K. A., & Kastner, D. L. (2014). Genetics, genomics, and their relevance to pathology and therapy. *Best Pract Res Clin Rheumatol, 28*(2), 175-189. doi:10.1016/j.berh.2014.05.001

ONS. (2011). *Ethnicity and National Identity in England and Wales 2011*. London.

ONS. (2017). *Overview of the UK population: July 2017*. London.

Orandi, A. B., Vogel, T. P., Keppel, M. P., Utterson, E. C., & Cooper, M. A. (2017). Azathioprine-Associated Complete NK Cell Deficiency. *J Clin Immunol*. doi:10.1007/s10875-017-0414-6

Orange, J. S., & Biron, C. A. (1996). An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. *J Immunol, 156*(3), 1138-1142.

Ottaviani, C., Nasorri, F., Bedini, C., de Pita, O., Girolomoni, G., & Cavani, A. (2006). CD56brightCD16(-) NK cells accumulate in psoriatic skin in response to CXCL10 and CCL5 and exacerbate skin inflammation. *Eur J Immunol, 36*(1), 118-128. doi:10.1002/eji.200535243

Pando, M. J., Gardiner, C. M., Gleimer, M., McQueen, K. L., & Parham, P. (2003). The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. *J Immunol, 171*(12), 6640-6649.

Papoutsis, N. G., Abdel-Naser, M. B., Altenburg, A., Orawa, H., Kotter, I., Krause, L., . . . Zouboulis, C. C. (2006). Prevalence of Adamantiades-Behçet's disease in Germany and the municipality of Berlin: results of a nationwide survey. *Clin Exp Rheumatol, 24*(5 Suppl 42), S125.

Parham, P., Norman, P. J., Abi-Rached, L., & Guethlein, L. A. (2011). Variable NK cell receptors exemplified by human KIR3DL1/S1. *J Immunol, 187*(1), 11-19. doi:10.4049/jimmunol.0902332

Park, S. H., Park, K. S., Seo, Y. I., Min, D. J., Kim, W. U., Kim, T. G., . . . Kim, H. Y. (2002). Association of MICA polymorphism with HLA-B51 and disease severity in Korean patients with Behçet's disease. *J Korean Med Sci, 17*(3), 366-370. doi:10.3346/jkms.2002.17.3.366

Pedersen, B. K., & Beyer, J. M. (1986). A longitudinal study of the influence of azathioprine on natural killer cell activity. *Allergy, 41*(4), 286-289.

Pelak, K., Need, A. C., Fellay, J., Shianna, K. V., Feng, S., Urban, T. J., . . . Alter, G. (2011). Copy number variation of KIR genes influences HIV-1 control. *PLoS Biol, 9*(11), e1001208. doi:10.1371/journal.pbio.1001208

Peruzzi, M., Parker, K. C., Long, E. O., & Malnati, M. S. (1996). Peptide sequence requirements for the recognition of HLA-B*2705 by specific natural killer cells. *J Immunol, 157*(8), 3350-3356.

Petrushkin, H., Hasan, M. S., Stanford, M. R., Fortune, F., & Wallace, G. R. (2015). Behçet's Disease: Do Natural Killer Cells Play a Significant Role? *Front Immunol, 6*, 134. doi:10.3389/fimmu.2015.00134

Picco, P., Porfirio, B., Gattorno, M., Buoncompagni, A., Falcini, F., Cusano, R., . . . Seri, M. (2002). MICA gene polymorphisms in an Italian paediatric series of juvenile Behçet disease. *Int J Mol Med, 10*(5), 575-578.

Piga, M., & Mathieu, A. (2011). Genetic susceptibility to Behçet's disease: role of genes belonging to the MHC region. *Rheumatology (Oxford), 50*(2), 299-310. doi:10.1093/rheumatology/keq331

Pittoni, V., Vaglio, S., Magrini, L., Accorinti, M., Pivetti-Pezzi, P., Girelli, G., & Valesini, G. (2003). Polymorphism of the Duffy erythrocyte chemokine receptor in Italian patients with Behçet's disease. *Rheumatol Int, 23*(3), 116-120. doi:10.1007/s00296-002-0269-9

Prince, H. E., Ettenger, R. B., Dorey, F. J., Fine, R. N., & Fahey, J. L. (1984). Azathioprine suppression of natural killer activity and antibody-dependent cellular cytotoxicity in renal transplant recipients. *J Clin Immunol, 4*(4), 312-318.

Rajagopalan, S., & Long, E. O. (2012). KIR2DL4 (CD158d): An activation receptor for HLA-G. *Front Immunol, 3*, 258. doi:10.3389/fimmu.2012.00258

Raulet, D. H. (2004). Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat Immunol, 5*(10), 996-1002. doi:10.1038/ni1114

Raulet, D. H. (2006). Missing self recognition and self tolerance of natural killer (NK) cells. *Semin Immunol, 18*(3), 145-150. doi:10.1016/j.smim.2006.03.003

Remmers, E. F., Cosan, F., Kirino, Y., Ombrello, M. J., Abaci, N., Satorius, C., . . . Gul, A. (2010). Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behçet's disease. *Nat Genet, 42*(8), 698-702. doi:10.1038/ng.625

Ricci-Vitiani, L., Vacca, A., Potolicchio, I., Scarpa, R., Bitti, P., Sebastiani, G., . . . Sorrentino, R. (2000). MICA gene triplet repeat polymorphism in patients with HLA-B27 positive and negative ankylosing spondylitis from Sardinia. *J Rheumatol, 27*(9), 2193-2197.

Riera-Mestre, A., Martinez-Yelamos, S., Martinez-Yelamos, A., Ferrer, I., Pujol, R., & Vidaller, A. (2010). Clinicopathologic features and outcomes of neuro-Behçet disease in Spain: a study of 20 patients. *Eur J Intern Med, 21*(6), 536-541. doi:10.1016/j.ejim.2010.08.003

Robinson, J., Halliwell, J. A., Hayhurst, J. D., Flicek, P., Parham, P., & Marsh, S. G. (2015). The IPD and IMGT/HLA database: allele variant databases. *Nucleic Acids Res, 43*(Database issue), D423-431. doi:10.1093/nar/gku1161

Robinson, J., Waller, M. J., Parham, P., Bodmer, J. G., & Marsh, S. G. (2001). IMGT/HLA Database--a sequence database for the human major histocompatibility complex. *Nucleic Acids Res, 29*(1), 210-213.

Rodacki, M., Milech, A., & de Oliveira, J. E. (2006). NK cells and type 1 diabetes. *Clin Dev Immunol, 13*(2-4), 101-107. doi:10.1080/17402520600877182

Saadoun, D., Wechsler, B., Desseaux, K., Le Thi Huong, D., Amoura, Z., Resche-Rigon, M., & Cacoub, P. (2010). Mortality in Behçet's disease. *Arthritis Rheum, 62*(9), 2806-2812. doi:10.1002/art.27568

Sakaguchi, T., Ibe, M., Miwa, K., Kaneko, Y., Yokota, S., Tanaka, K., & Takiguchi, M. (1997). Binding of 8-mer to 11-mer peptides carrying the anchor residues to slow assembling HLA class I molecules (HLA-B*5101). *Immunogenetics, 45*(4), 259-265.

Sakane, T., Takeno, M., Suzuki, N., & Inaba, G. (1999). Behçet's disease. *N Engl J Med, 341*(17), 1284-1291. doi:10.1056/NEJM199910213411707

Salvarani, C., Boiardi, L., Mantovani, V., Olivieri, I., Ciancio, G., Cantini, F., . . . Viggiani, M. (2001). Association of MICA alleles and HLA-B51 in Italian patients with Behçet's disease. *J Rheumatol, 28*(8), 1867-1870.

Salvarani, C., Pipitone, N., Catanoso, M. G., Cimino, L., Tumiati, B., Macchioni, P., . . . Boiardi, L. (2007). Epidemiology and clinical course of Behçet's disease in the Reggio Emilia area of Northern Italy: a seventeen-year population-based study. *Arthritis Rheum, 57*(1), 171-178. doi:10.1002/art.22500

Sanjanwala, B., Draghi, M., Norman, P. J., Guethlein, L. A., & Parham, P. (2008). Polymorphic sites away from the Bw4 epitope that affect interaction of Bw4+ HLA-B with KIR3DL1. *J Immunol, 181*(9), 6293-6300.

Sant, S. M., Kilmartin, D., & Acheson, R. A. (1998). HLA antigens in familial Behçet's disease in Ireland. *Br J Rheumatol, 37*(11), 1250-1251.

Saruhan-Direskeneli, G., Uyar, F. A., Cefle, A., Onder, S. C., Eksioglu-Demiralp, E., Kamali, S., . . . Gul, A. (2004). Expression of KIR and C-type lectin receptors in Behçet's disease. *Rheumatology, 43*(4), 423-427. doi:DOI 10.1093/rheumatology/keh063

Saunders, P. M., Pymm, P., Pietra, G., Hughes, V. A., Hitchen, C., O'Connor, G. M., . . . Vivian, J. P. (2016). Killer cell immunoglobulin-like receptor 3DL1 polymorphism defines distinct hierarchies of HLA class I recognition. *J Exp Med, 213*(5), 791-807. doi:10.1084/jem.20152023

Saunders, P. M., Vivian, J. P., Baschuk, N., Beddoe, T., Widjaja, J., O'Connor, G. M., . . . Brooks, A. G. (2015). The interaction of KIR3DL1*001 with HLA class I molecules is dependent upon molecular microarchitecture within the Bw4 epitope. *J Immunol, 194*(2), 781-789. doi:10.4049/jimmunol.1402542

Sensi, A., Gavioli, R., Spisani, S., Balboni, A., Melchiorri, L., Menicucci, A., . . . Baricordi, O. R. (1991). HLA B51 antigen associated with neutrophil hyper-reactivity. *Dis Markers, 9*(6), 327-331.

Seoudi, N., Bergmeier, L. A., Drobniewski, F., Paster, B., & Fortune, F. (2015). The oral mucosal and salivary microbial community of Behçet's syndrome and recurrent aphthous stomatitis. *J Oral Microbiol, 7*, 27150. doi:10.3402/jom.v7.27150

Sezer, F. N. (1953a). Cultivation of virus of epidemic keratoconjunctivitis on chorioallantoic membrane of fertile egg. *AMA Arch Ophthalmol, 49*(3), 293-302.

Sezer, F. N. (1953b). The isolation of a virus as the cause of Behçet's diseases. *American journal of ophthalmology, 36*(3), 301-315.

Shih, W. W., Ellison, G. W., Myers, L. W., Durkos-Smith, D., & Fahey, J. L. (1982). Locus of selective depression of human natural killer cells by azathioprine. *Clin Immunol Immunopathol, 23*(3), 672-681.

Shimizu, S., Chen, K. R., Ikemoto, K., & Han-Yaku, H. (1998). Abrupt onset of severe Behçet's disease: preceding oral ulceration is not essential for diagnosis. *Br J Dermatol, 139*(1), 160-161.

Shriver, M. D., Parra, E. J., Dios, S., Bonilla, C., Norton, H., Jovel, C., . . . Kittles, R. A. (2003). Skin pigmentation, biogeographical ancestry and admixture mapping. *Hum Genet, 112*(4), 387-399. doi:10.1007/s00439-002-0896-y

Skotte, L., Korneliussen, T. S., & Albrechtsen, A. (2013). Estimating individual admixture proportions from next generation sequencing data. *Genetics, 195*(3), 693-702. doi:10.1534/genetics.113.154138

Sobel, J. D., Haim, S., Shafrir, A., & Gellei, B. (1973). Cutaneous hyperreactivity in Behçet's disease. *Dermatologica, 146*(6), 350-356.

Sun, A., Chang, J. G., Chu, C. T., Liu, B. Y., Yuan, J. H., & Chiang, C. P. (1998). Preliminary evidence for an association of Epstein-Barr virus with pre-ulcerative oral lesions in patients with recurrent aphthous ulcers or Behçet's disease. *J Oral Pathol Med, 27*(4), 168-175.

Sun, A., Chang, J. G., Kao, C. L., Liu, B. Y., Wang, J. T., Chu, C. T., . . . Chiang, C. P. (1996). Human cytomegalovirus as a potential etiologic agent in recurrent aphthous ulcers and Behçet's disease. *J Oral Pathol Med, 25*(5), 212-218.

Suzuki, Y., Hoshi, K., Matsuda, T., & Mizushima, Y. (1992). Increased peripheral blood gamma delta+ T cells and natural killer cells in Behçet's disease. *J Rheumatol, 19*(4), 588-592.

Takemoto, Y., Naruse, T., Namba, K., Kitaichi, N., Ota, M., Shindo, Y., . . . Kimura, A. (2008). Re-evaluation of heterogeneity in HLA-B*510101 associated with Behçet's disease. *Tissue Antigens, 72*(4), 347-353. doi:10.1111/j.1399-0039.2008.01111.x

Takeno, M., Kariyone, A., Yamashita, N., Takiguchi, M., Mizushima, Y., Kaneoka, H., & Sakane, T. (1995). Excessive function of peripheral blood neutrophils from patients with Behçet's disease and from HLA-B51 transgenic mice. *Arthritis Rheum, 38*(3), 426-433.

Takeno, M., Shimoyama, Y., Kashiwakura, J., Nagafuchi, H., Sakane, T., & Suzuki, N. (2004). Abnormal killer inhibitory receptor expression on natural killer cells in patients with Behçet's disease. *Rheumatol Int, 24*(4), 212-216. doi:10.1007/s00296-003-0352-x

Takeuchi, M., Kastner, D. L., & Remmers, E. F. (2015). The immunogenetics of Behçet's disease: A comprehensive review. *J Autoimmun*. doi:10.1016/j.jaut.2015.08.013

Takeuchi, M., Mizuki, N., Meguro, A., Ombrello, M. J., Kirino, Y., Satorius, C., . . . Remmers, E. F. (2017). Dense genotyping of immune-related loci implicates host responses to microbial exposure in Behçet's disease susceptibility. *Nat Genet, 49*(3), 438-443. doi:10.1038/ng.3786

Tanaka, T., Yamakawa, N., Koike, N., Suzuki, J., Mizuno, F., & Usui, M. (1999). Behçet's disease and antibody titers to various heat-shock protein 60s. *Ocul Immunol Inflamm, 7*(2), 69-74.

Taner, S. B., Pando, M. J., Roberts, A., Schellekens, J., Marsh, S. G., Malmberg, K. J., . . . Brodsky, F. M. (2011). Interactions of NK cell receptor KIR3DL1*004 with chaperones and conformation-specific antibody reveal a functional folded state as well as predominant intracellular retention. *J Immunol, 186*(1), 62-72. doi:10.4049/jimmunol.0903657

Tao, S. D., He, Y. M., Ying, Y. L., He, J., Zhu, F. M., & Lv, H. J. (2014). KIR3DL1 genetic diversity and phenotypic variation in the Chinese Han population. *Genes Immun, 15*(1), 8-15. doi:10.1038/gene.2013.55

Tasci, B., Direskeneli, H., Serdaroglu, P., Akman-Demir, G., Eraksoy, M., & Saruhan-Direskeneli, G. (1998). Humoral immune response to mycobacterial heat shock protein (hsp)65 in the cerebrospinal fluid of neuro-Behçet patients. *Clin Exp Immunol, 113*(1), 100-104.

Taylor, J., Glenny, A. M., Walsh, T., Brocklehurst, P., Riley, P., Gorodkin, R., & Pemberton, M. N. (2014). Interventions for the management of oral ulcers in Behçet's disease. *Cochrane Database Syst Rev, 9*, CD011018. doi:10.1002/14651858.CD011018.pub2

Tian, W., Boggs, D. A., Ding, W. Z., Chen, D. F., & Fraser, P. A. (2001). MICA genetic polymorphism and linkage disequilibrium with HLA-B in 29 African-American families. *Immunogenetics, 53*(9), 724-728. doi:10.1007/s00251-001-0392-x

Tian, W., Boggs, D. A., Uko, G., Essiet, A., Inyama, M., Banjoko, B., . . . Fraser, P. A. (2003). MICA, HLA-B haplotypic variation in five population groups of sub-Saharan African ancestry. *Genes Immun, 4*(7), 500-505. doi:10.1038/sj.gene.6364017

Tian, W., Li, L. X., Wang, F., Luo, Q. Z., Yan, M. Y., Yu, P., . . . Cao, Y. (2006). MICA-STR, HLA-B haplotypic diversity and linkage disequilibrium in the Hunan Han population of southern China. *Int J Immunogenet, 33*(4), 241-245. doi:10.1111/j.1744-313X.2006.00605.x

Tian, Z., Gershwin, M. E., & Zhang, C. (2012). Regulatory NK cells in autoimmune disease. *J Autoimmun, 39*(3), 206-215. doi:10.1016/j.jaut.2012.05.006

Tojo, M., Zheng, X., Yanagihori, H., Oyama, N., Takahashi, K., Nakamura, K., & Kaneko, F. (2003). Detection of herpes virus genomes in skin lesions from patients with Behçet's disease and other related inflammatory diseases. *Acta Derm Venereol, 83*(2), 124-127.

Tong, H. V., Toan, N. L., Song, L. H., Bock, C. T., Kremsner, P. G., & Velavan, T. P. (2013). Hepatitis B virus-induced hepatocellular carcinoma: functional roles of MICA variants. *J Viral Hepat, 20*(10), 687-698. doi:10.1111/jvh.12089

Treusch, M., Vonthein, R., Baur, M., Gunaydin, I., Koch, S., Stubiger, N., . . . Kotter, I. (2004). Influence of human recombinant interferon-alpha2a (rhIFN-alpha2a) on

altered lymphocyte subpopulations and monocytes in Behçet's disease. *Rheumatology (Oxford), 43*(10), 1275-1282. doi:10.1093/rheumatology/keh311

Trundley, A., Frebel, H., Jones, D., Chang, C., & Trowsdale, J. (2007). Allelic expression patterns of KIR3DS1 and 3DL1 using the Z27 and DX9 antibodies. *Eur J Immunol, 37*(3), 780-787. doi:10.1002/eji.200636773

Trydzenskaya, H., Juerchott, K., Lachmann, N., Kotsch, K., Kunert, K., Weist, B., . . . Babel, N. (2013). The genetic predisposition of natural killer cell to BK virus-associated nephropathy in renal transplant patients. *Kidney Int, 84*(2), 359-365. doi:10.1038/ki.2013.59

Turner, M. J., Delay, M. L., Bai, S., Klenk, E., & Colbert, R. A. (2007). HLA-B27 up-regulation causes accumulation of misfolded heavy chains and correlates with the magnitude of the unfolded protein response in transgenic rats: Implications for the pathogenesis of spondylarthritis-like disease. *Arthritis Rheum, 56*(1), 215-223. doi:10.1002/art.22295

Tursen, U., Gurler, A., & Boyvat, A. (2003). Evaluation of clinical findings according to sex in 2313 Turkish patients with Behçet's disease. *Int J Dermatol, 42*(5), 346-351.

Ugolini, S., Arpin, C., Anfossi, N., Walzer, T., Cambiaggi, A., Forster, R., . . . Vivier, E. (2001). Involvement of inhibitory NKRs in the survival of a subset of memory-phenotype CD8+ T cells. *Nat Immunol, 2*(5), 430-435. doi:10.1038/87740

Vacher-Coponat, H., Brunet, C., Moal, V., Loundou, A., Bonnet, E., Lyonnet, L., . . . Paul, P. (2006). Tacrolimus/mycophenolate mofetil improved natural killer lymphocyte reconstitution one year after kidney transplant by reference to cyclosporine/azathioprine. *Transplantation, 82*(4), 558-566. doi:10.1097/01.tp.0000229390.01369.4a

Varol, A., Seifert, O., & Anderson, C. D. (2010). The skin pathergy test: innately useful? *Arch Dermatol Res, 302*(3), 155-168. doi:10.1007/s00403-009-1008-9

Vendelbosch, S., de Boer, M., van der Heijde, D., van den Berg, T. K., van Gaalen, F. A., & Kuijpers, T. W. (2015). KIR3DL1 and KIR3DL2 gene copy number variation in axial spondyloarthritis. *Tissue Antigens, 85*(6), 497-498. doi:10.1111/tan.12563

Venstrom, J. M., Gooley, T. A., Spellman, S., Pring, J., Malkki, M., Dupont, B., . . . Hsu, K. C. (2010). Donor activating KIR3DS1 is associated with decreased acute GVHD in unrelated allogeneic hematopoietic stem cell transplantation. *Blood, 115*(15), 3162-3165. doi:10.1182/blood-2009-08-236943

Verity, D. H., Marr, J. E., Ohno, S., Wallace, G. R., & Stanford, M. R. (1999). Behçet's disease, the Silk Road and HLA-B51: historical and geographical perspectives. *Tissue Antigens, 54*(3), 213-220.

Wallace, G. R., Verity, D. H., Delamaine, L. J., Ohno, S., Inoko, H., Ota, M., . . . Vaughan, R. W. (1999). MIC-A allele profiles and HLA class I associations in Behçet's disease. *Immunogenetics, 49*(7-8), 613-617.

Walzer, T., Dalod, M., Robbins, S. H., Zitvogel, L., & Vivier, E. (2005). Natural-killer cells and dendritic cells: "l'union fait la force". *Blood, 106*(7), 2252-2258. doi:10.1182/blood-2005-03-1154

Weiss-Steider, B., Soto-Cruz, I., Martinez-Campos, C. A., & Mendoza-Rincon, J. F. (2011). Expression of MICA, MICB and NKG2D in human leukemic myelomonocytic and cervical cancer cells. *J Exp Clin Cancer Res, 30*, 37. doi:10.1186/1756-9966-30-37

Whitley, D., Goldberg, S. P., & Jordan, W. D. (1999). Heat shock proteins: a review of the molecular chaperones. *Journal of vascular surgery : official publication, the*

Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter, 29(4), 748-751.

Wildner, G., & Thurau, S. R. (1994). Cross-reactivity between an HLA-B27-derived peptide and a retinal autoantigen peptide: a clue to major histocompatibility complex association with autoimmune disease. *Eur J Immunol, 24*(11), 2579-2585. doi:10.1002/eji.1830241103

Xavier, J. M., Shahram, F., Sousa, I., Davatchi, F., Matos, M., Abdollahi, B. S., . . . Oliveira, S. A. (2015). FUT2: filling the gap between genes and environment in Behçet's disease? *Ann Rheum Dis, 74*(3), 618-624. doi:10.1136/annrheumdis-2013-204475

Yabuhara, A., Yang, F. C., Nakazawa, T., Iwasaki, Y., Mori, T., Koike, K., . . . Komiyama, A. (1996). A killing defect of natural killer cells as an underlying immunologic abnormality in childhood systemic lupus erythematosus. *J Rheumatol, 23*(1), 171-177.

Yabuki, K., Mizuki, N., Ota, M., Katsuyama, Y., Palimeris, G., Stavropoulos, C., . . . Ohno, S. (1999). Association of MICA gene and HLA-B*5101 with Behçet's disease in Greece. *Invest Ophthalmol Vis Sci, 40*(9), 1921-1926.

Yamaguchi, Y., Takahashi, H., Satoh, T., Okazaki, Y., Mizuki, N., Takahashi, K., . . . Kuwana, M. (2010). Natural killer cells control a T-helper 1 response in patients with Behçet's disease. *Arthritis Res Ther, 12*(3), R80. doi:10.1186/ar3005

Yang, P., Fang, W., Meng, Q., Ren, Y., Xing, L., & Kijlstra, A. (2008). Clinical features of chinese patients with Behçet's disease. *Ophthalmology, 115*(2), 312-318 e314. doi:10.1016/j.ophtha.2007.04.056

Yang, S. S., Park, K. M., Park, Y. J., Kim, Y. W., Do, Y. S., Park, H. S., . . . Kim, D. I. (2013). Peripheral arterial involvement in Behçet's disease: an analysis of the

results from a Korean referral center. *Rheumatol Int, 33*(8), 2101-2108. doi:10.1007/s00296-013-2700-9

Yasuoka, H., Yamaguchi, Y., Mizuki, N., Nishida, T., Kawakami, Y., & Kuwana, M. (2008). Preferential activation of circulating CD8+ and gammadelta T cells in patients with active Behçet's disease and HLA-B51. *Clin Exp Rheumatol, 26*(4 Suppl 50), S59-63.

Yawata, M., Yawata, N., Draghi, M., Little, A. M., Partheniou, F., & Parham, P. (2006). Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. *J Exp Med, 203*(3), 633-645. doi:10.1084/jem.20051884

Yazici, H., Fresko, I., & Yurdakul, S. (2007). Behçet's syndrome: disease manifestations, management, and advances in treatment. *Nat Clin Pract Rheumatol, 3*(3), 148-155. doi:10.1038/ncprheum0436

Yazici, H., Pazarli, H., Barnes, C. G., Tuzun, Y., Ozyazgan, Y., Silman, A., . . . et al. (1990). A controlled trial of azathioprine in Behçet's syndrome. *N Engl J Med, 322*(5), 281-285. doi:10.1056/NEJM199002013220501

Yazici, H., Tuzun, Y., Pazarli, H., Yurdakul, S., Ozyazgan, Y., Ozdogan, H., . . . Muftuoglu, A. U. (1984). Influence of age of onset and patient's sex on the prevalence and severity of manifestations of Behçet's syndrome. *Ann Rheum Dis, 43*(6), 783-789.

Yoon, J. H., Shin, S., Park, M. H., Song, E. Y., & Roh, E. Y. (2010). HLA-A, -B, -DRB1 allele frequencies and haplotypic association from DNA typing data of 7096 Korean cord blood units. *Tissue Antigens, 75*(2), 170-173. doi:10.1111/j.1399-0039.2009.01418.x

Yurdakul, S., Gunaydin, I., Tuzun, Y., Tankurt, N., Pazarli, H., Ozyazgan, Y., & Yazici, H. (1988). The prevalence of Behçet's syndrome in a rural area in northern Turkey. *Journal of Rheumatology, 15*(5), 820-822.

Yurdakul, S., & Yazici, H. (2008). Behçet's syndrome. *Best Pract Res Clin Rheumatol, 22*(5), 793-809. doi:10.1016/j.berh.2008.08.005

Zhang, Z., Wiseman, N., & Wilms, S. (2013). *Jin guì yào lüè : translation and commentaries - Essential prescriptions of the golden cabinet*. Taos, N.M.: Paradigm Publications ;

Distributed by Redwing Books.

Zheng, Z., Sohn, S., Ahn, K. J., Bang, D., & Cho, S. B. (2015). Serum reactivity against herpes simplex virus type 1 UL48 protein in Behçet's disease patients and a Behçet's disease-like mouse model. *Acta Derm Venereol, 95*(8), 952-958. doi:10.2340/00015555-2127

Zhou, Q., Wang, H., Schwartz, D. M., Stoffels, M., Park, Y. H., Zhang, Y., . . . Aksentijevich, I. (2016). Loss-of-function mutations in TNFAIP3 leading to A20 haploinsufficiency cause an early-onset autoinflammatory disease. *Nat Genet, 48*(1), 67-73. doi:10.1038/ng.3459

Zouboulis, C. C. (1999). Epidemiology of Adamantiades-Behçet's disease. *Ann Med Interne (Paris), 150*(6), 488-498.

Zouboulis, C. C. (2002). Benediktos Adamantiades and his forgotten contributions to medicine. *Eur J Dermatol, 12*(5), 471-474.

Zouboulis, C. C., Kotter, I., Djawari, D., Kirch, W., Kohl, P. K., Ochsendorf, F. R., . . . Orfanos, C. E. (1997). Epidemiological features of Adamantiades-Behçet's disease in Germany and in Europe. *Yonsei Med J, 38*(6), 411-422.